PB-230 344

THE INTERLABORATORY PRECISION TEST. AN EIGHT LABORATORY EVALUATION OF THE PROVISIONAL ALGAL ASSAY PROCEDURE BOTTLE TEST

Charles M. Weiss, et al

North Carolina University
Chapel Hill, North Carolina

October 1971

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PB 230 344

Provisional Algal Assay Procedure
EPA-Water Quality Office Project-16010DQT

THE INTERLABORATORY PRECISION TEST
An Eight Laboratory Evaluation of the Provisional Algal Assay Procedure Bottle Test

Charles M. Weiss
Professor of Environmental Biology

and

Ronald W. Helms, Director
Computing Services
Department of Biostatistics
School of Public Health

With the Technical Assistance of:
Mrs. Gail Marshall-Research Associate
Department of Environmental Sciences and Engineering

Department of Environmental Sciences and Engineering
School of Public Health
University of North Carolina at Chapel Hill
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FOREWORD

The investigation described herein represents a concerted effort by eight laboratories to evaluate the precision of the "Bottle Test" of the Provisional Algal Assay Procedure in determining the growth response of a test alga to various nutrient concentrations. In this investigation the growth response of the test alga was compared in various dilutions of a synthetic culture medium. In a subsequent interlaboratory precision test, algal growth responses in natural lake water samples were studied; one common water sample was provided to all eight participating laboratories by the National Eutrophication Research Program and another was collected by each laboratory from a lake in its geographic area. The results of the latter investigation will be published in the near future. We feel that this approach of using interlaboratory precision tests for evaluating and refining an assay procedure is rather unique.

A special note of appreciation should be extended to Professor Charles M. Weiss and Dr. Ronald W. Helms, and members of their respective staffs at the University of North Carolina at Chapel Hill for their efforts in compiling and statistically evaluating the data submitted to them by the participating laboratories.

Thomas E. Maloney, Deputy Chief
National Eutrophication
Research Program
National Environmental Research
Center, Corvallis, Oregon
Environmental Protection Agency

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Preface

This report summarizes the efforts of a substantial number of technical and professional individuals who in eight "teams" over a period of several months independantly followed an assay protocol. It is perhaps of considerable significance that whereas interlab variation might be considered high, for reasons as yet not defined, the precision within each laboratory was remarkably good. The authors of this report and their associates and assistants who helped to digest and assemble the data wish to express their sincere appreciation to all the participating laboratories for their wholehearted cooperation.

All raw data as submitted on the reporting form were transferred to data cards. Major analytical steps were suitably programmed and computations carried out on the Triangle Universities Computation Center IBM 360/75 computer. Many of the statistical computations were done by Mrs. Mary Donelan and Mrs. Mary Flanagan of the Department of Biostatistics.

Summary

In order to establish the validity of an algal assay procedure for the determination of algal nutrient levels in surface waters a suitable protocol was designed and followed by eight laboratories. This group consisted of one government laboratory, four university laboratories and three industrial laboratories. The basic procedure was to evaluate by use of the "bottle" or batch test the precision and reproducibility of the growth response of one test organism, Selenastrum capricornutum, in four media of varying nutrient strength. The medium was originally defined for the PAAP test and modified slightly in subsequent evaluations. The test media of this experiment were all dilutions of the PAAP medium.

In addition to following the growth of the test alga in the four "mandatory" media, several of the participating laboratories examined growth response in the basic medium minus specific nutrient elements. The test protocol also permitted evaluation of growth controlling factors such as continuous shaking versus handswirling, comparison of the use of the hemacytometer versus Coulter Counter in determining cell numbers, and the levels of correlation between cell mass and cell number as determined by optical density, chlorophyll flourescence and total algal carbon.

The unusual nature of this evaluation in the numbers of laboratories participating and magnitude of the test size has permitted the statistical analysis to be developed to the extent that the results will set new guidelines for future assays of this type.

Conclusions

1. Growth response of S. capricornutum as determined by various parameters, e.g., cell no/ml, log cell no/ml, wgt-mg/ml and cell wgt mg/10^6 cells, showed coefficients of variation of the mean, averaged for the eight laboratories, that varied from about 3 to nearly 240 percent. This range of variation related not only to the parameter measured but also the nutrient strength of the medium. The determination of cell mass (21 days growth) in the B medium (now considered the reference medium) had a c. v. of mean of 27.3%, whereas cell no/ml was 42.3%, cell wgt/10^6 cells, 36.2% and log cell no/ml 2.9%.

2. Comparison of counting methods, hemacytometer versus Coulter Counter, at 13 and 21 days growth, showed no evidence that one procedure consistently resulted in lower coefficients of variation of the mean than the other.

3. Comparison of growth rates in the four mandatory media, over several time sequences, showed that for the three media with highest nutrient strength maximum growth rate was in the 3-5 day period. Variation among the laboratories ranged from 27-50%. It would appear that the usefulness of the bottle assay would be enhanced if growth rate determinations were made for 0-3, 3-5, 5-7 and 7-9 day intervals and used as another measure of response to nutrient strength.

4. Where it was possible to compare growth response of the stock algal cultures to local cultures of the same species, no consistent difference was apparent in any parameter analyzed.

5. Comparison of growth response in cultures hand swirled daily versus those grown on a shaking table, 110 oscillations/min, showed no consistent difference.

6. Removal of P and N from the standard medium (B) and replacement with smaller quantities resulted in growth response proportionally less. The presence or absence of Fe in this medium was considerably less significant than P or N in terms of growth response.

7. The very high correlation coefficients found for algal mass-optical density, algal mass-total carbon and algal mass-fluorescence would indicate that these alternate measurements can be related to algal mass and used for this determination with an accuracy of more than 95%.

8. The regression analysis of nutrient strength and algal yield indicated that each laboratory in its use of the assay consistently and significantly produced growth response that was linearly related to

nutrient strength. However, differences among the laboratories were also consistently significant, e.g., the slopes of the regressions for each lab were different.

9. It has been shown by this interlaboratory comparison that algal assays may be defined for (a) estimating the difference between the effects of two or more treatments, media of different strength and (b) estimating the total number of cells that may be grown under standard conditions. The former may be called a comparative assay and the latter a descriptive assay. Statistical considerations for these two types of assay are different.

10. Descriptive algal assay experiments have relatively poor accuracy; it is not practical to produce precise estimates of the biomass which would be produced under a set of "ideal" conditions. The loss of accuracy is a result of large systematic differences among laboratories. Moreover, within any one laboratory it is not possible to estimate the accuracy of experimental results because the same systematic error is manifest in all measurements within a laboratory. However, comparative algal assays, experiments for estimating the difference between biomasses which would be produced under two sets of ideal conditions, in comparison are quite precise. Small differences in strengths of media can be detected. Or, a sample of unknown strength can be accurately assayed in comparison to media of known strengths, and various labs will show close agreement on such comparative assays.

Comparisons between laboratories will be of a lower order of precision until the systematic variables that establish the exact conditions of growth are better understood and their limits defined.

PAAP EVALUATION

INTERLABORATORY PRECISION TEST

Introduction

In the development of an assay procedure which requires evaluation of the growth response of some organism to a particular set of growth conditions, it is important to establish limits of variability that might be expected when all growth conditions, except the unknown, are kept as constant as possible within experimental limitations. The several laboratories involved in the development of the algal assay procedure for the evaluation of nutrient levels in surface waters decided in the spring of 1970 that many of the variables of laboratory growth conditions were sufficiently defined, when applied to the bottle or batch test, to carry out a comparative experiment by the eight participating laboratories to establish limits of variability. This experiment would require all participating laboratories to follow a set of prescribed conditions and procedures using common materials and cultures with their results being subjected to statistical analysis for the purposes of answering the question of what is the inherent variability that might be expected in an algal assay bottle test.

Experimental Protocol

Basic to a test of precision of this assay was elimination of variables that might be introduced using different batches of chemicals, for the nutrient media as well as cultures of the same species of algae but of different age or origin. These variables were eliminated by having all laboratories prepare their test media from a common batch of reagents supplied by one laboratory and all using a fresh subculture of Selenastrum capricornutum obtained from the

National Eutrophication Research Program, Corvallis, Oregon. In addition to a basic set of growth experiments which would be designated mandatory, it was optional at any of the laboratories to carry out parallel experiments using local chemicals or local cultures of the test alga which may have originated at an earlier date from the National Eutrophication Research Program but due to local conditions of transfer and handling, may have undergone physiological change of unknown characteristic. The set of instructions for the experimental protocol transmitted to all participating laboratories, eight in number, is attached to this document as Appendix A.

The laboratories participating in this comparison test to establish precision of the algal assay procedure are listed in the following table.

TABLE 1

Participating Laboratories

1. Department of Environmental Sciences and Engineering
   University of North Carolina
   Chapel Hill, North Carolina

2. Water Resources Center
   University of Wisconsin
   Madison, Wisconsin

3. Environmental Water Quality Research Laboratory
   Procter and Gamble Company
   Cincinnati, Ohio

4. School of Engineering
   University of California
   Irvine, California

5. Chemical Research and Development Center
   FMC Corporation
   Princeton, New Jersey

6. National Eutrophication Research Program
   Corvallis, Oregon

7. Lever Brothers Research Center
   Edgewater, New Jersey

8. Richmond Field Station
   University of California
   Berkeley, California

In the following discussion of test results the laboratory number shown in the preceding table will be used in nearly all instances rather than the actual name of the laboratory. The particular sequencing of these numbers has no significance except indicating the order in which the data were received at the University of North Carolina, the laboratory designated to carry out the statistical evaluation. The manner in which the evaluation was carried out by the various laboratories is shown in Table 2. This degree of involvement in the evaluation reflects the particular local interest, manpower availability, as well as other local factors at the particular period of the evaluation. All laboratories, however, carried out the mandatory comparisons. The statistical analysis will therefore focus primarily on these although the extra tests carried out by several of the laboratories permits other conclusions, derived from these additional tests. Deviations from experimental factors such as period of incubation, temperature, illumination, shaking rates, as described in the protocol sent to all laboratories are shown in Table 3. These generally illustrate the usual laboratory problems that arise due to difficulties of temperature control, shaking rates, as well as making cell count determinations on days other than those specified in the protocol.

Parameters and Statistical Analysis Used in Evaluation of the Algal Assay Batch Test

The basic eight laboratory evaluation consisted of a comparison of the media designated A, B, C, D, made up with the UNC chemicals and tested by evaluating the growth of the Corvallis strain of Selenastrum capricornutum. The evaluation of the growth response in these four media was based on the ultimate yield as dry weight as well as direct cell counts (See Appendix B). In determining cell numbers comparisons were also made between the results obtained by use of the hemacytometer as well as the Coulter Counter. Since dry weight determination was only possible at the end of the experiment whereas

Table 2

Summary of Laboratory Procedures as Followed by the Participating Laboratories

	Laboratory							
	UNC(1)	WIS(2)	P&G(3)	IRV(4)	FMC(5)	COR(6)	LEVER(7)	BERK(8)
Culture Source								
Corvallis	X	X	X	X	X	X	X	X
Local	X	X[a]	X[d]				X[c]	
Chemical Source								
UNC	X	X	X	X	X	X	X	X
Local					X[b]		X[c]	
Media								
A - D	X	X[a]	X	X	X	X	X	X
E		X[a]	X		X			
F		X[a]	X		X			
G		X[a]	X		X			
H		X[a]	X		X			
I		X[a]	X		X			
J		X[a]	X		X			
K			X		X			
L			X		X			
M			X		X			
N			X		X			
P			X		X			
Q			X					
R			X					
S			X					
T			X					
U			X					

[a]Evaluated at 18 & 21 days only; Local culture used in media A-D only.

[b]Used only with medium designated B.

[c]Used in B medium only: Corvallis algae with local chemicals
Local algae with UNC chemicals

[d]Used in media A-D only.

Table 3

Summary of Individual Laboratory Deviations from IPT Protocol

		Specified Range		
	Incubation Time (Days) When Specified Range Exceeded	Temperature 22-26° C.	Illumination 380-420 f.c.	Shaking Rate 110 oscill./min.
Laboratory		Exceeded Values		
UNC	17 21	--- ---	431 451	--- ---
U. of Wis.	all (swirled flasks only)	---	368	---
P & G	3	21.6	---	---
Irvine	3-17 21	--- 21-22	--- ---	165 165
FMC	---	---	---	---
Corvallis	21 (1st Run only)	---	431	---
Lever Bros.	5 7 21	29.9 27.4 30.1	--- --- ---	--- --- ---
Berkeley	---	---	---	---

cell numbers could be determined throughout the period of the growth response, other cell mass related measurements were also utilized, such as optical density of the algal suspension, fluorescence and algal carbon. The pH of the algal suspensions was also measured at various incubation times throughout the growth period. The changing growth rates as nutrient materials were utilized and converted to cell mass were computed for various time sequences in the total growth period and subjected to statistical analysis to establish coefficients of variation. Where feasible, regression analyses were carried out relating nutrient strength and cell yield. Correlation coefficients were determined between weight yield and optical density, fluorescence and algal carbon.

The algal assay tests carried out on the optional media, E-J (using UNC chemicals and Corvallis algae) were analyzed with reference to growth yield and compared to the basic medium B. This growth response was similarly measured as cell number and cell mass. Growth rates were computed and compared to medium B for various incubation periods.

Wherever a comparison was made between the use of local algal cultures and the fresh subculture, obtained from the National Eutrophication Research Program, similar parameters were compared to establish whether any variation in growth yield could be significantly associated with one or the other cultures. These comparisons include cell number, dry weight, weight per cell as well as growth rates.

Statistical Interpretation

In the analysis of replicate samples as carried out in the interlaboratory precision evaluation, there are frequent occasions in which one or more values among the replicate observation deviates from the mean to an extent that some test should be applied to examine its validity for inclusion in the replicate set of observations. The questionable value may,

for instance, lie three or four standard deviations or further from the mean of all values. Such a value, called an outlier, may be a peculiarity not at all typical of the rest of the data. Outliers should be submitted to examination to determine if the reason for its peculiarity can be determined and rules used to establish whether it should be included or rejected.

It is not a wise procedure to automatically reject a value if it should lie three or four standard deviations from the mean. Rules have been proposed for rejecting such outliers after which the data are reanalyzed with these observations removed. Sometimes an outlier may provide information which other data points do not in that it may arise from an unusual combination of circumstances which may be of vital interest and require further investigation rather than rejection. As a general rule outliers should not be rejected out of hand unless they can be traced to causes such as errors in recording observations or in apparatus malfunction. If a definite cause cannot be located for an outlier it can be tested for validity for inclusion in the data set according to procedures outlined in Appendix B. Such tests for outliers have been applied to questionable data points in the analysis of the data submitted by the eight participating laboratories.

In biological assays with their inherent variability replicate samples are required to establish the magnitude of the standard deviation. The number in the replicate set will be determined by several factors including the precision desired from the assay. Some guidelines for establishing the optimum number in the replicate are described in Appendix C.

Results

Mandatory Tests

The comparative analysis of results from the mandatory media, as submitted by the eight laboratories was based on the several growth parameters. These

included determination of pH of the media at various incubation times (a measure of the utilization of CO_2) and cell multiplication determined through the use of two counting techniques, as indicated previously, and as part of the analysis the transformation of the cell number to $\log_{10}$ cell/ml to improve the "normal distribution" of the actual cell counts obtained from replicate flasks. Finally, at the end of the growth period the dry weight of cells produced in 21 days incubation was determined. A series of figures and tables have been prepared to summarize this information. Figures 1 and 2 present the cell number/ml following 21 days incubation for media A, B, C and D as reported by the eight participating laboratories, the mean values for the replicate flasks indicated by a point, with a bar extending above and below this point representing the 95% confidence limit for the particular replicate set. Although there might appear to be a trend indicated in media A and B of an increase in cell number from left to right, Lab 1 to 8, this is essentially a happenstance. In this particular presentation it simply shows that in medium A Lab 1 results were somewhat lower than the average and Lab 8 results somewhat higher. What is particularly interesting is the value presented by Lab 5 for media B and C as compared to the averages presented from the other laboratories. This deviation was recognized as possibly some basic problem in these two media at this particular laboratory and consequently a rerun was made of these two media to establish whether this was a real deviation or one which was due to some peculiar circumstances at the time of the first test. The results of the second run are described by an open circle with a dashed line representing the 95% confidence limit. It is apparent that an anomaly had occured in the first circumstance, whereas the second set of data from Lab 5 for these two media approaches the average value to a much greater degree. The close conformation with the averages of the other laboratories emphasizes the reliability and reproducibility of the assay if all external

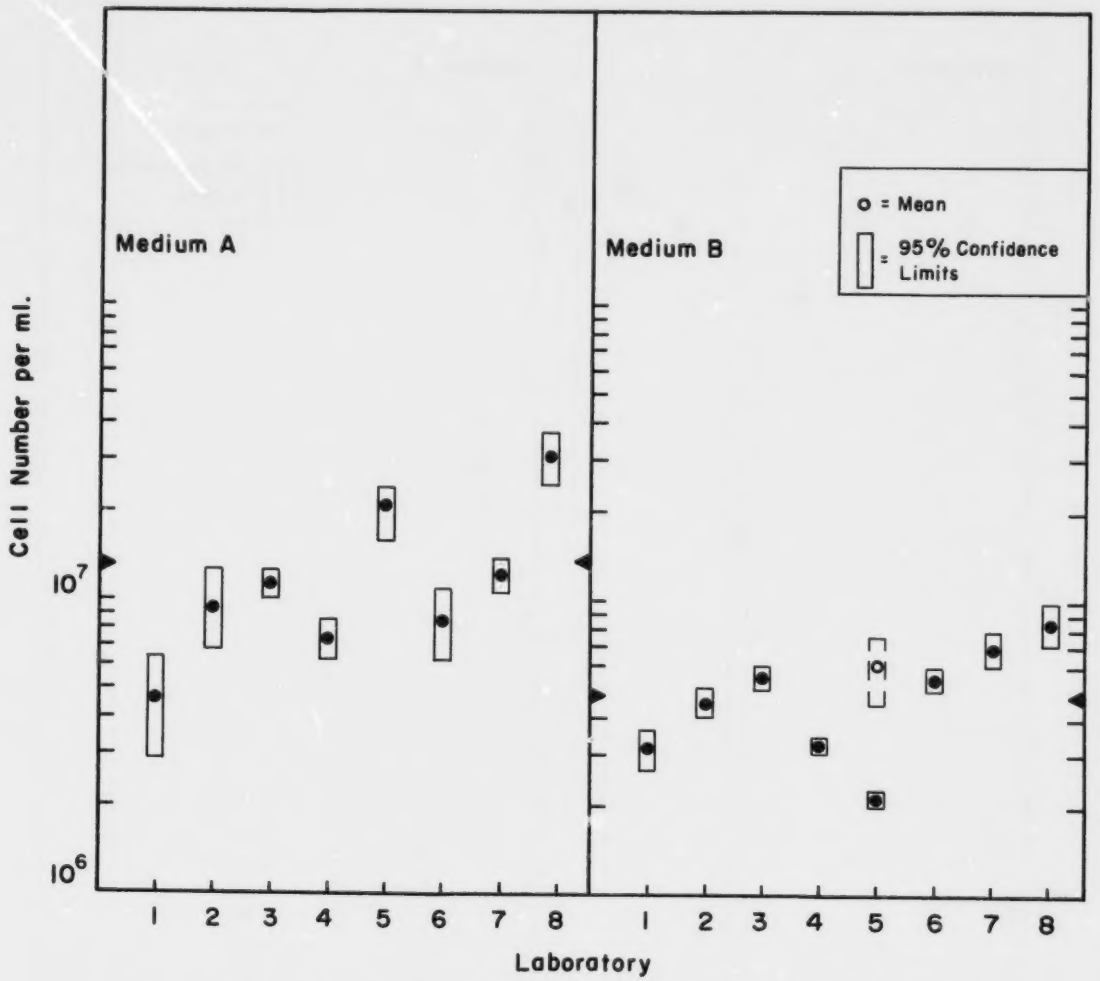

Fig. 1. Cell number per ml., means and 95% Confidence Limits, 21 days incubation, media A and B. ▶ Average of eight laboratories. Dashed bar rerun of lab. 5.

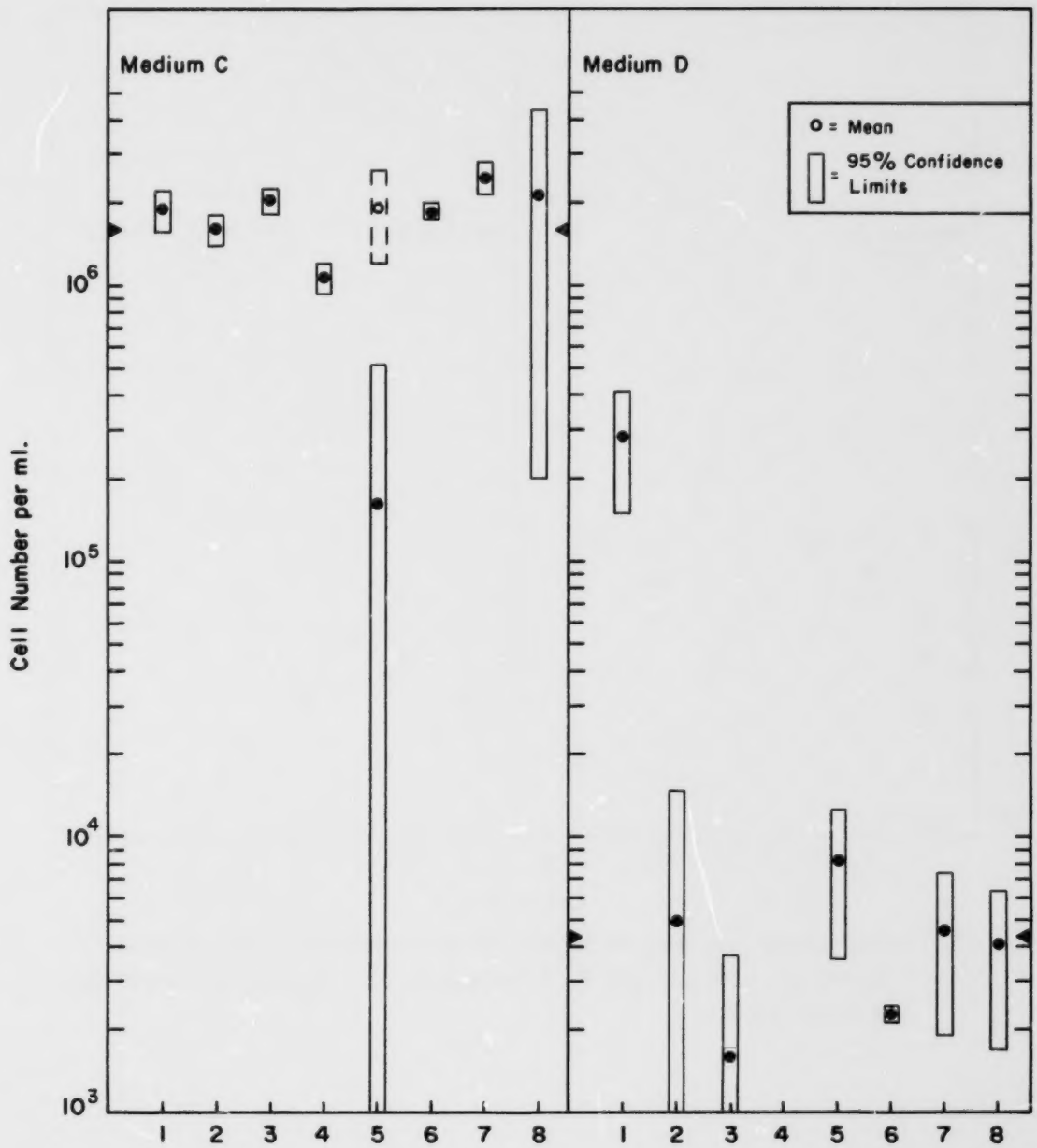

Fig. 2. Cell number per ml., means and 95% confidence limits, 21 days incubation, media C and D. ▶ Average of eight laboratories. Dashed bar rerun of lab 5.

conditions are carefully controlled. The larger deviation from the average, shown in medium D, the lowest nutrient concentration of the mandatory media and broader bands of the 95% confidence limit is a characteristic of this assay as nutrient levels decrease.

Figure 3 presents for the four mandatory media the cell number transformed to the $\log_{10}$ and presented in similar fashion with the point depicting the mean value and a bar describing the 95% confidence limit. Aside from reducing the degree of scatter as might be expected with a log transformation, the results are similar to that described by the data in Figures 1 and 2.

At the end of 21 days incubation suitable samples of the cell mass produced in each of the four media were dried and weighed. These results are presented in Figure 4. It is immediately apparent as compared to the direct cell counts that probably due to the difficulty of determining accurately small quantities of cell mass, a greater degree of variability is shown by the results. This, for some reason, is particularly striking with the strongest medium, which on the average produced the greatest cell mass but also exhibited the greatest interlaboratory variation. As the strength of medium decreased, particularly with media B and C the degree of variation between laboratories, even though unit mass was lower, proved considerable. With medium D, the amount of cell mass formed in 21 days in some instances was so small that it was not practical to obtain accurate mass determinations, and in several laboratories no data were reported for this growth parameter.

A third parameter that lent itself to interlaboratory comparison was the calculated value for the unit cell mass. The data as presented are computed as dry weight for 10^6 cells. Thus if the quality of the medium was reproducible and growth conditions uniform throughout the eight laboratories, the unit mass

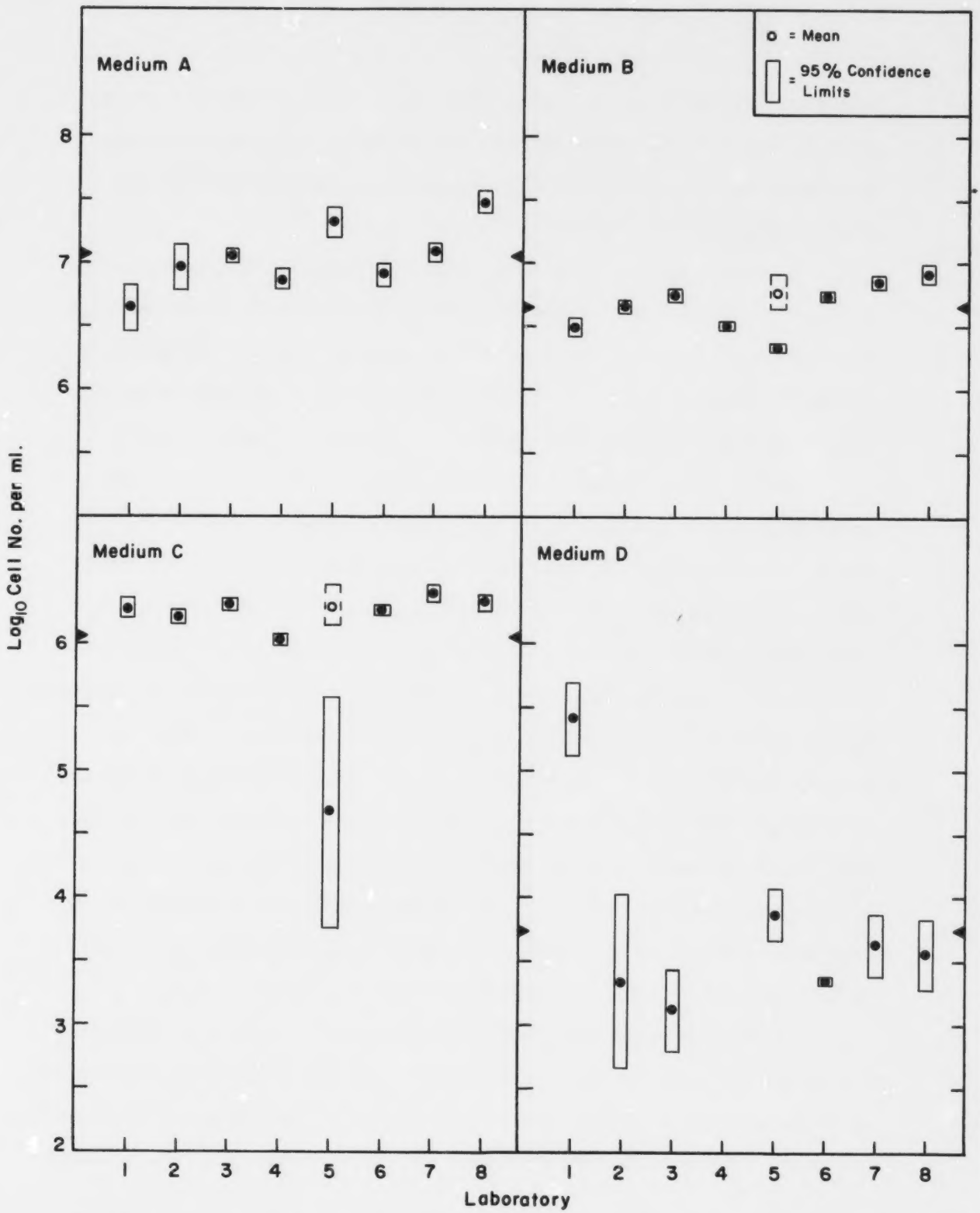

Fig. 3. Log_{10} cell number per ml., means and 95% confidence limits, 21 days incubation, media A, B, C and D. ▶ Average of eight laboratories. Dashed bar, rerun of lab. 5.

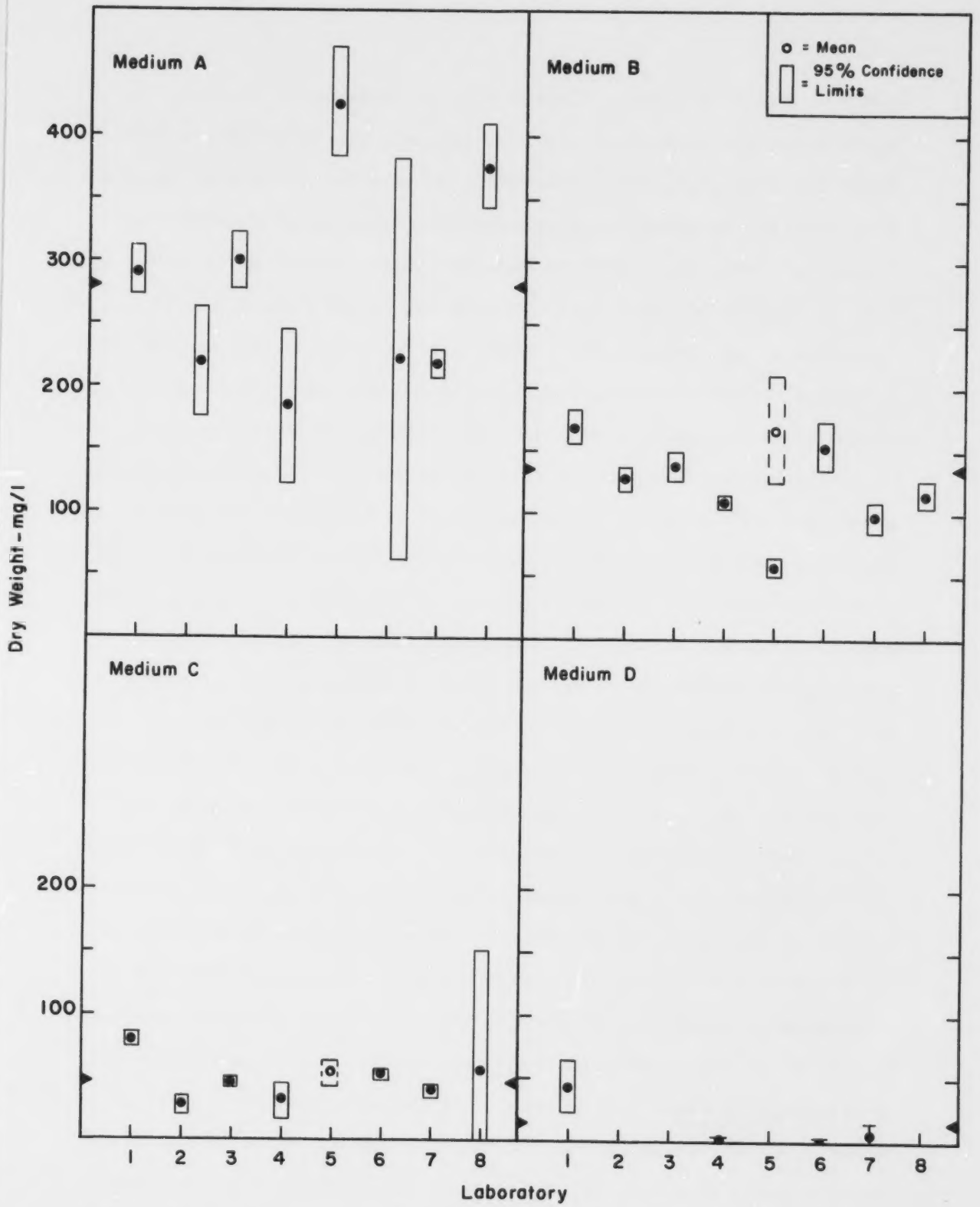

Fig. 4. Dry weight, means and 95% confidence limits, 21 days incubation, media A, B, C and D. ▶ Average of eight laboratories. Dashed bar rerun of lab. 5.

should be fairly consistent. These results are presented in Figure 5. Due to the difficulty of obtaining accurate total cell mass determination from medium D no computation for this parameter was made for this medium. By some peculiarity of the laboratory environment there is a consistent pattern indicated that Laboratory 1 produced cells of a higher unit weight in media A, B and C. This was quite marked for both media A and B and to a lesser degree for medium C. All laboratories in their tests in medium C, when compared with each other, exhibited a greater degree of variability than either media A or B except for the distinctly heavier unit mass developed at Laboratory 1.

Although the determination of pH of the media during the course of cell growth provided a reference to the rapidity of cell synthesis, utilization of CO_2 and consequent increase in pH, basic difficulties in measuring the pH of a cell suspension often introduced a degree of variation which may have exaggerated the percent variation of this growth parameter. The pH values as reported by the laboratories at various stages of incubation were averaged for the five replicate samples and percent variation calculated in each instance (Table 4). In some cases the stronger media, A and B, showed rapid shifts in pH by five days growth rising to values greater than pH 10 and remaining high for the remaining incubation period. However, this was not characteristic of all laboratory determinations; in some instances the rise was much slower and did not achieve the high values as reported, for instance, in Laboratories 4 and 5. A similar pattern of response was seen in medium B, which may be indicative of some degree of combination of artifacts introduced by the measuring instrument, as well as the rate at which CO_2 was being reabsorbed from the atmosphere to reduce the pH to a more neutral range. High pH values were not evident in medium C, and the minimal growth response of medium D produced no real change over the period of growth, although markedly different pH values were recorded

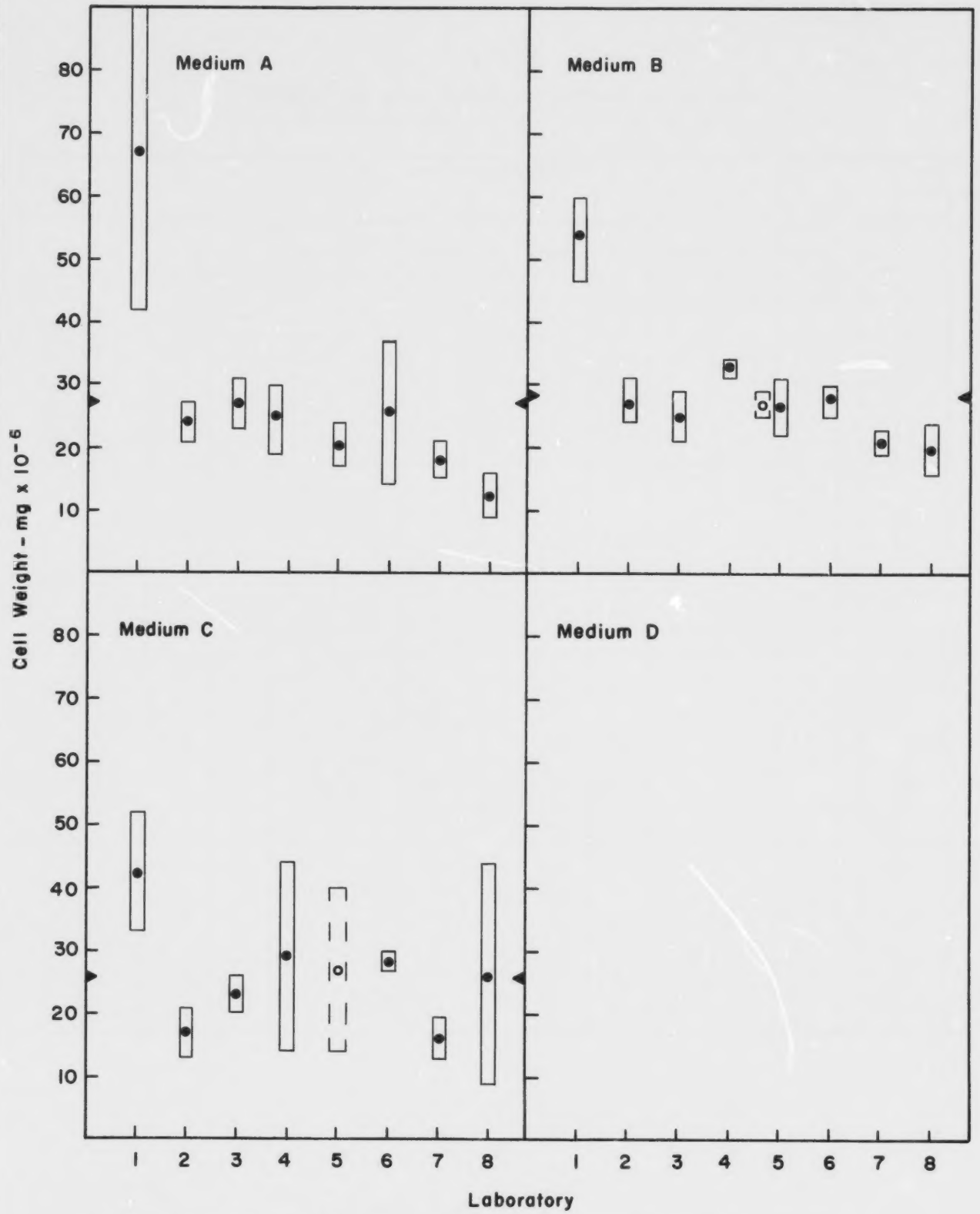

Fig. 5. Dry weight per 10^6 cells, means and 95% confidence limits, 21 days incubation, media A, B, C and D ▶ Average of eight laboratories. Dashed bar rerun of lab 5.

Table 4

Mean pH and Coefficient of Variation of the Mean
at Specified Incubation Times, Media A-D

Medium	Lab No.	Incubation Time - Days 3		5		7		9		13		17		21	
		$\bar{x}$	%Var.	$\bar{x}$	%Var.	$\bar{x}$	%Var.	$\bar{x}$	%Var.	$\bar{x}$	%Var.	$\bar{x}$	%Var.	$\bar{x}$	%Var.
A	1	7.4	2.3	---	---	---	---	10.1	0.2	---	---	---	---	9.6	0.7
	2	7.3	0.8	---	---	8.2	1.1	---	---	---	---	---	---	8.5	2.3
	3	7.8	0.3	---	---	---	---	10.0	0.2	9.9	0.4	9.5	0.4	8.9	0.7
	4	7.9	0.3	10.4	0.2	10.6	0.2	10.5	0.5	10.3	0.6	9.9	1.2	9.8	0.2
	5	7.9	0.0	10.2	0.4	9.8	0.4	10.2	0.6	9.5	0.5	---	---	8.7	0.3
	6	8.1	1.4	9.7	0.3	9.6	0.4	9.4	0.4	9.3	0.3	9.1	0.4	8.8	0.6
	7	8.1	0.3	---	---	---	---	10.4	0.4	---	---	---	---	9.2	0.4
	8	7.0	0.4	7.9	0.6	8.1	0.9	8.8	1.0	8.2	0.6	8.5	0.7	---	---
B	1	7.0	3.8	---	---	---	---	9.6	2.6	---	---	---	---	8.2	4.7
	2	7.2	0.9	---	---	8.1	0.4	---	---	---	---	---	---	6.7	0.3
	3	7.4	0.0	---	---	---	---	9.3	0.6	7.7	0.3	7.6	0.7	7.5	0.3
	4	7.4	0.3	10.4	0.3	10.5	0.2	10.3	0.2	8.2	2.0	7.9	0.6	8.2	0.8
	5	7.1	0.0	7.7	0.5	7.3	0.6	7.3	0.3	7.2	0.4	---	---	7.4	0.3
	6	7.7	0.5	9.1	0.4	9.4	0.4	9.6	0.4	8.5	0.8	8.0	0.5	7.5	0.4
	7	7.7	1.0	---	---	---	---	10.3	0.5	---	---	---	---	8.2	0.6
	8	6.8	1.1	7.7	2.2	7.8	0.5	8.2	1.8	7.8	1.1	8.2	0.7	---	---
C	1	6.7	0.6	---	---	---	---	8.5	3.8	---	---	---	---	7.0	1.3
	2	6.6	2.8	---	---	6.8	0.0	---	---	---	---	---	---	6.0	0.3
	3	7.2	0.0	---	---	---	---	7.3	0.9	7.2	0.0	7.1	0.4	7.1	0.3
	4	7.2	0.3	8.1	4.5	7.6	3.6	7.9	0.6	---	---	7.4	0.0	7.6	0.7
	5	7.3	0.0	7.5	0.3	7.3	0.3	7.4	0.3	7.3	0.6	---	---	7.7	1.8
	6	7.4	0.3	8.0	0.4	7.3	0.5	7.9	0.5	7.5	0.8	7.4	0.7	7.2	0.6
	7	7.3	0.3	---	---	---	---	7.5	0.0	---	---	---	---	7.6	0.0
	8	6.4	5.5	7.7	1.3	7.4	2.0	7.4	5.4	7.7	4.6	7.4	1.4	---	---
D	1	5.4	1.1	---	---	---	---	6.5	4.7	---	---	---	---	6.6	1.6
	2	5.2	3.7	---	---	4.8	2.1	---	---	---	---	---	---	3.9	0.8
	3	6.9	0.6	---	---	---	---	6.5	1.9	6.6	2.9	6.5	2.1	6.3	3.0
	4	7.2	0.3	6.9	0.0	---	---	7.1	0.5	---	---	---	---	7.1	0.9
	5	6.8	0.0	7.1	0.0	6.7	0.4	6.8	0.4	6.8	0.3	---	---	7.6	1.2
	6	7.3	0.5	7.8	0.5	7.3	0.7	7.5	0.5	7.1	0.7	7.0	0.7	6.9	0.7
	7	6.5	2.2	---	---	---	---	6.6	1.9	---	---	---	---	6.7	2.1
	8	5.6	1.1	7.1	1.4	7.0	1.0	7.0	0.7	7.0	1.2	7.2	1.3	---	---

for medium D by the several laboratories. This medium also exhibited a higher degree of variation in pH values between replicate samples, partially reflecting the large variation in growth response noted in this dilute medium as well as inherent machine variability.

In the final assessment the results of all eight laboratories can be considered as a test unit and the degree of variation or coefficient of variation computed to establish the expected order of precision for this type of assay. These data, assembled in Table 5, indicate a certain degree of consistency among various measuring parameters as well as an indication of which ones would be preferable. The dry weight analysis indicated that in the three strongest media, A, B and C, the coefficient of variation of the mean described an algal assay that has variation of 27% to 37%. The most dilute medium, D, resulted in cell mass production with variation of over 160% among laboratories. Cell count comparison shows for media B and C variation of 43% to 44%, slightly higher for medium A, 65%, and again a very extreme variation of 238% for medium D. The log transformation of the cell count by the very nature of the mathematics reduces the variation so that media A and B show a 3% to 4% variation among laboratories, slightly less than 10% for medium C and about 20% for medium D. The unit weight per cell analysis confirms the same variation found with the cell count, with the C and B media showing the smallest variation of 34% to 36% and the A medium rising to 61% variation. This relationship between the strength of media and degree of variation that one expected for this type of asaay raises some significant questions concerning its routine applicability. This follows from the rationale that waters of unknown nutrient strength may vary to a considerable degree from the nutrient strength of media B and C and thus assay with a higher order of variation. The laboratory carrying out such an assay must examine with considerable care variation within standard media that are run in parallel to unknown samples.

Table 5

Average Values and Coefficient of Variation of the Mean
in Mandatory Media as Determined by the Eight Laboratories

Media	A		B		C		D	
	Average	%Var.*	Average	%Var.	Average	%Var.	Average	%Var.
21 Days Growth								
cell no./ml	1.32×10^7	65.5	4.95×10^6	42.3	1.65×10^6	43.9	4.35×10^4	238.9
$\log_{10}$cell no./ml	7.04	3.7	6.65	2.9	6.05	9.4	3.75	20.4
wgt. mg/l	280.4	30.5	133.0	27.3	47.4	36.8	13.0	160.4
cell wgt.mg/10^6cells	27.5	61.1	29.5	36.2	26.1	33.7	---	---

* % Var. describes variability of mean cell counts from lab to lab

Comparison of Other Growth Measurements

It was possible through the efforts applied in this test by Laboratory 3 to establish for the growth response in media A-D a comparison between the standard growth parameters of cell number, log cell, dry weight and weight per cell, and measurements of optical density, chlorophyll, fluorescence and algal carbon and to ascertain whether any of the latter had levels of variation that compared with the standard procedures. These additional determinations were made only at 21 days, so their coefficients of variation should appropriately be only compared with the other parameters at the same time period. All three measures of cell mass in the higher nutrient strength media, A, B and C, showed a significantly lesser degree of variation than was obtained with the direct cell counts or dry weight determination (Table 6). Even in the more dilute media, where higher degrees of variation were frequently found, it is particularly noteworthy that the fluorescence determination and algal carbon had less than one half the variation experienced with direct cell counts. Averaging the three stronger media, a 1% variation could be expected in optical density measurements, about 2% for measurements of chlorophyll fluorescence and 2.2% for algal carbon. It should be noted that these lower degrees of variation experienced with the above methods are due in good part to the lower sensitivity or ability to respond to small differences as compared, for instance, to direct cell counts.

Interlaboratory Variation

A multi-laboratory comparison provides an opportunity to establish levels of variation that might be expected on the average in replicate series established for the particular assay. It also provides an opportunity to determine whether a particular method of cell counting, such as in this case by hemacytometer or Coulter Counter, was significantly different in establishing lesser or greater degrees of variation among replicate samples. Table 7 presents for two days in

Table 6

A Comparison of Coefficient of Variation of the Mean of Various Algal Growth Parameters as Determined in Laboratory 3*

All at 21 days

	Medium				
	A	B	C	D	Av.(A,B,C)
Dry Weight	2.77%	3.10%	2.10%	----	2.66%
Cell No.	3.57	3.71	3.50	37.5	3.59
Log Cell No.	0.22	0.25	0.25	3.9	0.24
Wgt./Cell	5.70	5.61	4.71	----	5.34
Optical Density	1.14	0.74	1.27	100.0	1.05
Fluorescence	2.5	2.7	0.73	17.5	1.99
Algal Carbon	1.62	2.72	2.41	16.7	2.25

*Corvallis culture, S. capricornutum, UNC chemicals, illumination and shaking, standard

Table 7

Coefficient of Variation in Cell Number Per Milliliter and Log_{10} Cell Number Per Milliliter, 13 and 21 Days Incubation, Media A-D

A Description of the Average Variability in Each Laboratory

Coefficient of Variation - Cell Number/ml.

Lab No.	13 days				21 days				Count Method[a]
	A	B	C	D	A	B	C	D	
1	25.5%	8.1%	1.4%	37.2*%	13.5%	5.8%	6.3%	17.0*%	H C*
2	16.3	5.2	4.8	----	11.6	3.7	3.9	72.7	H
3	2.4	5.7	3.2	28.6	3.6	3.7	3.5	37.5	H
4	13.4	8.1 (5.8)	7.4 (8.9)	----	5.4	1.6 (9.8)	4.4 (13.0)	----	H
5	10.5	2.7	69.9	19.9	9.3	1.9	78.4	19.8	C
6	4.0	6.0	1.6	5.2	2.1	2.8	1.9	2.1	C
7	6.9	9.0	3.5	12.6	4.8	4.8	4.7	21.0	C
8	25.4	4.3	13.7	22.1	6.3	5.9	18.9	20.7	C
Average	13.0	6.5	5.5	20.9	6.6	4.7	7.0	27.2	

Coefficient of Variation - Log_{10} Cell Number/ml.

Lab No.	13 days				21 days				Count Method[a]
	A	B	C	D	A	B	C	D	
1	1.8%	0.6%	0.1%	3.6*%	0.9%	0.4%	0.4%	1.9*%	H C*
2	1.5	0.4	0.3	---	0.9	0.2	0.3	7.3	H
3	0.2	0.3	0.2	3.1	0.2	0.3	0.3	3.9	H
4	1.0	0.5 (0.3)	0.6 (0.6)	---	0.4	0.1 (0.6)	0.3 (0.9)	---	H
5	0.6	0.2	5.3	2.0	0.5	0.1	7.1	2.0	C
6	0.3	0.4	0.1	0.7	0.1	0.2	0.1	0.3	C
7	0.5	0.6	0.2	1.3	0.3	0.3	0.3	2.5	C
8	1.3	0.3	0.9	2.2	0.4	0.4	1.3	2.8	C
Average	0.9	0.4	0.4	1.8	0.5	0.3	0.5	2.9	

( ) nos. used in averaging, the repeat determination of Lab 5

[a]H - hemacytometer; C - Coulter Counter

*Counts in D media by Coulter Counter

the growth sequence, 13 and 21 days, the percent variation in cell number and log cell number/ml for the mandatory media, A, B, C and D. In this table the two counting procedures are grouped together, and just by coincidence Laboratories 1 through 4 utilized a hemacytometer and 5 through 8 the Coulter Counter. In similar fashion a comparison of dry weight assay at 21 days is shown in Table 8 as well as the percent variation for cell weight per 10^6 cells.

There is one consistent data pattern that emerges from these computations. In media A, B and C the overall average variation, regardless of the technique used for establishing cell response in the particular medium, was less than 10%. The best of the averages, less than 1%, was found when cell counts were analyzed by the log transformation. Of perhaps of even more significance, with respect to the utilization of an algal assay on unknown materials, was the pattern of variation in terms of variation between replicates as seen when the four media are compared in a manner which relates to their being either a multiple or a fraction of the nutrient strength standard or B medium. Why the B medium and its particular concentration of macro and micro nutrient elements provided the optimum conditions in which variation was minimal is not exactly clear. The choice of concentration was based on a recognition that the prior standard medium, PAAP, designated as the A medium in this experiment, was apparently too strong to use as a reference medium, and its strength was reduced to 30% of the original. It would appear that at the reduced strength optimum growth conditions that related to uniformity of cell multiplication appeared to have been established, and variation within replicates was minimized.

Comparison of Variation Between Replicates In Which Cell Numbers Are Established By the Use of Coulter Counter or Hemacytometer

Basic to the determination of cell growth in either reference or unknown samples some technique must be used to determine with reasonable accuracy changes in cell number. This may be either by direct enumeration using a hemacytometer,

Table 8

Coefficient of Variation of the Mean in Dry Weight (mg/l) and Weight (mg) Per 10^6 Cells, 21 Days Incubation, Media A-D

Coefficient of Variation - Dry Weight (mg/l)

Lab No.	Medium A	B	C	D
1	2.4%	2.9%	2.4%	17.9%
2	8.8	2.7	8.7	---
3	2.8	3.1	2.1	---
4	12.2	1.4	16.3	22.7
5	3.7	4.1	---	---
6	5.6	4.3	2.2	66.7
7	1.8	2.8	4.2	60.4
8	2.7	2.2	13.7	---
Average	5.0	2.9	7.0	41.9

Coefficient of Variation - Weight per 10^6 Cells

Lab No.	Medium A	B	C	D
1	13.2%	4.3%	8.0%	51.9%
2	4.8	4.4	8.6	---
3	5.7	5.6	4.7	---
4	8.0	1.8	18.2	---
5	6.4	6.1	---	---
6	3.5	3.0	2.1	65.9
7	5.8	3.3	7.5	66.5
8	8.8	7.4	5.3	---
Average	7.0	4.4	7.7	61.4

by particle determination using a Coulter Counter or other procedures which relate by measurement of some other property of the cell number such as chlorophyll fluorescence, algal carbon or changes in optical density due to increase in cell number. The techniques employed by the several laboratories permitted a comparative analysis to be made between the Coulter Counter and hemacytometer to determine whether one or the other would show a clear indication of higher order of accuracy or lower percent variation between replicates. This comparison was possible with the four mandatory media, and the data for this comparison are shown in Table 9.

As has already been noted each of the media have different orders of variation due to the nature of cell response in the particular nutrient concentration. There is no consistent pattern except that medium B tended to have a lower level of variation in replicate series than the other media. When the growth response in medium B was compared, as determined by Coulter Counter or hemacytometer, it appeared that at 13 days growth there was slightly lower variation for the Coulter Counter than at 21 days as compared to the hemacytometer. The differences among the other media showed no clear evidence that one or the other counting procedures was distinctly better. However, as shown at the bottom of Table 9, if all variations for the four media are averaged, A-D and then the three strongest media, A, B and C, it would seem to indicate that at 13 days there was clearly no distinction as to whether one counting technique was more accurate or minimized to a greater degree variation between replicates. At 21 days the difference in variation between techniques, while slightly larger, was hardly significant.

Specific Growth Rates

It has been long argued that the response of an algal culture to specific nutrient elements in a medium is best defined by the growth rate in the logarithmic phase of multiplication rather than the ultimate cell mass produced

Table 9

Comparison of Average Coefficient of Variation of the Mean in Measurements Involving the Coulter Counter or Hemacytometer, 13 and 21 Days Incubation, Media A-D

Type of Measurement	Medium	Counting Technique			
		Coulter Counter		Hemacytometer	
		13 Days	21 Days	13 Days	21 Days
cell no./ml	A	11.7%	5.6%	14.4%	8.5%
	B	5.5 (6.2)	3.8 (5.8)	9.4	3.7
	C	22.2 (6.9)	26.0 (9.6)	3.1	4.5
	D	19.4	16.1	14.3	55.1
log cell no./ml	A	0.7	0.3	1.1	0.6
	B	0.4 (0.4)	0.3 (0.4)	0.7	0.2
	C	1.6 (0.4)	2.2 (0.7)	0.2	0.3
	D	2.0	1.9	1.5	5.6
weight/cell	A	---	6.1	---	7.9
	B	---	4.9	---	4.0
	C	---	4.9	---	9.9
	D	---	61.5	---	---

	Average	Coulter Counter		Hemacytometer	
		13 Days	21 Days	13 Days	21 Days
cell no./ml	A-D	11.0%	9.3%	10.3%	17.9%
	A-C	8.2	7.0	8.9	5.5

( ) values calculated using re-run data of Lab 5

within the medium. It is indicated that growth limiting factors are minimal in the log phase of growth and thus the characteristic response of the algae to the nutrient characteristics of the medium is best illustrated by the rate of multiplication during this period. Of course the measure of the rate depends upon the technique of cell number enumeration, the difference in cell number between the two time reference points and the total time elapsed. Thus the assay laboratory's contribution to this value depends upon a careful determination of cell numbers of some other cell-related observation at the two points in time to determine change in cell number for that period on the growth curve.

The interlaboratory test protocol called for cell number determinations at specific intervals; however, it was not possible in some laboratories to do these at the precise interval requested, and these are suitably footnoted in Table 10 in which the mean specific growth rates for the various time intervals are presented. The initial cell number value was the inoculum sample of algae, 1000 cells/ml, with the first cell count resulting from growth response being made at 3 days. The overall mean values for the eight laboratories clearly indicated that the maximum growth rate occured in the 3 to 5 day incubation period for media A and B. Medium C showed maximum growth rates in the 0 to 3 day period as well as from 3 to 5 days. Growth rates in medium D were quite variable among laboratories as well as in the overall mean for the several time intervals with the maximum growth rate in the 5 to 7 day period less than one half the maximum found in medium C and approximately one third the maximum growth rate of media B and A. Variation among laboratories, when the rates for each laboratory were compared with the overall mean, showed that the lowest percent variation systematically occured in the 0 to 3 day period of incubation, possibly because of the standardization of the zero day count. However, the percent variation of the growth rate in the 3 to 5 day period of incubation is

Table 10

Comparison of Mean Specific Growth Rates During Indicated Incubation Periods, Media A, B, C, D (UNC Chemicals, Corvallis Culture)

Medium A

Lab No.	Incubation Times (days) 0-3	3-5	5-7	7-9	9-13	13-17	17-21
1	1.10	1.11	0.68	0.13	0.12	0.13	0.08
2	1.39	1.03	0.22	0.19[a]	----	0.05[b]	0.01[c]
3	1.26	1.52	0.43	0.26	0.23	0.06	-0.01
4	1.62	1.19	0.25	0.18	0.17	0.03	0.00
5	1.68	0.82	0.99	0.34	0.11	0.04[d]	0.02[e]
6	1.20	1.59	0.12	0.34	0.38	0.06	0.03
7	1.40	1.30	0.23	0.36	0.03	0.31	0.02
8	0.69	1.96	-0.17	1.74	0.25	0.04	0.00
Overall Mean	1.29	1.31	0.34	0.48	0.19	0.10	0.02
Percent Variation	24.19	27.42	103.61	117.69	63.04	99.80	143.85
No. Labs Averaged	8	8	8	7	7	6	6

Medium B

Lab No.	Incubation Times (days) 0-3	3-5	5-7	7-9	9-13	13-17	17-21
1	1.32	0.64	1.01	0.30	0.08	-0.01	-0.02
2	1.39	1.04	0.40	0.06[a]	----	0.00[b]	0.02[c]
3	1.27	1.57	0.57	0.10	0.05	0.05	-0.01
4	1.39	1.54	0.34	0.11	0.00	0.02	-0.04
5	1.59	1.17	0.22	0.04	0.02	-0.01[d]	0.00[e]
6	1.04	1.48	0.88	0.20	0.07	0.01	0.01
7	1.46	1.55	0.38	-0.08	-0.01	0.20	0.01
8	0.72	2.07	0.86	0.31	0.06	0.02	0.01
Overall Mean	1.27	1.38	0.58	0.14	0.04	0.05	-0.01
Percent Variation	21.56	31.04	50.96	102.15	100.78	152.42	226.77
No. Labs Averaged	8	8	8	7	7	6	6

a 7-13 days
b 13-18 days
c 18-21 days
d 13-16 days
e 16-21 days
f 0-5 days
g 5-18 days

Table 10 (Cont'd)

Mean Specific Growth Rates

Medium C

Lab No.	Incubation Times (days)						
	0-3	3-5	5-7	7-9	9-13	13-17	17-21
1	1.22	0.78	1.01	0.19	0.01	-0.04[b]	0.01
2	1.29	1.13	0.18	0.01[a]	----	-0.01[b]	0.00[c]
3	1.32	1.56	0.16	0.02	0.02	0.01	0.01
4	1.37[f]	----	0.13	-0.07	0.01	0.00	-0.01
5	1.10	0.09	0.09	-0.08	0.18	-0.19	0.04[e]
6	0.67	0.82	1.38	0.42	0.06	0.00	0.01
7	1.45	1.50	0.19	0.02	-0.01	-0.03	0.04
8	0.50	1.28	0.66	0.59	0.37	-0.09	-0.00
Overall Mean	1.08	1.02	0.47	0.16	0.09	-0.02	0.01
Percent Variation	33.23	49.79	102.76	164.44	151.94	157.93	173.40
No. Labs Averaged	7	7	8	7	7	6	6

Medium D

Lab No.	Incubation Times (days)						
	0-3	3-5	5-7	7-9	9-13	13-17	17-21
1	0.48	-0.56	1.89	-0.93	0.29	0.49	0.04
2	----	----	-0.08[g]	----	----	----	-0.50[c]
3	0.00	0.00	0.11	0.07	-0.03	-0.05	0.07
4	----	----	----	----	----	----[d]	----
5	0.92	0.09	-0.23	0.01	-0.06	0.30[d]	-0.39[e]
6	0.30	0.04	0.02	-0.04	-0.04	-0.02	0.03
7	0.46	-0.11	0.01	0.26	-0.01	-0.09	0.03
8	0.00	0.20	0.74	-0.56	0.22	-0.09	0.00
Overall Mean	0.36	-0.06	0.42	0.20	0.06	0.05	0.03
Percent Variation	96.39	472.17	186.07	227.88	249.57	500.92	74.66
No. Labs Averaged	6	6	6	6	6	5	5

a 7-13 days
b 13-18 days
c 18-21 days
d 13-16 days
e 16-21 days
f 0-5 days
g 5-18 days

only slightly greater for the A and B media, a little more for the C medium, and of course there was tremendous variation found in the D medium.

The variation in mean specific growth rates for the various mandatory media is graphically presented in Figure 6 for the several growth periods. It is clearly indicated that if growth rate determinations are to be used as measure of response to nutrient characteristics of the particular water, this should be carried out in the first week of incubation with cell count determinations or other related parameters made at least on the third, fifth and seventh day to establish maximum rate for the particular medium or water.

Comparison of Continuous Shaking or Hand Swirling

The question as to whether batch culture, bottles or flasks, should be continually shaken or occasionally hand swirled during the period of incubation was evaluated by a comparative analysis that could be made of the two techniques of agitation, utilizing data obtained by Laboratory 2. Here comparable replicate sets were shaken and hand swirled using the standard algal culture and chemicals provided for the interlaboratory precision test. As shown in Figure 7, Laboratory 2 hand swirled cultures, media A, B and C, as contrasted to the growth rates in shaken flasks were slightly greater in the 0 to 3 day period. In the 3 to 5 day period growth rates were higher in shaken cultures than in hand swirled cultures. However, by the third time period, 5 to 7 days, two of the media, B and C, showed slightly greater growth rates in the hand swirled flask, whereas growth rates in medium A were slightly greater in the shaken flasks. Subsequently, growth rates slowed and were inconclusive as to whether there was any difference between the shaken and hand swirled cultures. CO_2 limitation can be significant in a medium of high nutrient concentration. The rate of CO_2 transfer from the atmosphere to the medium might be slightly better in shaken flasks and thus allow for the somewhat better growth in the A medium during the log phase growth.

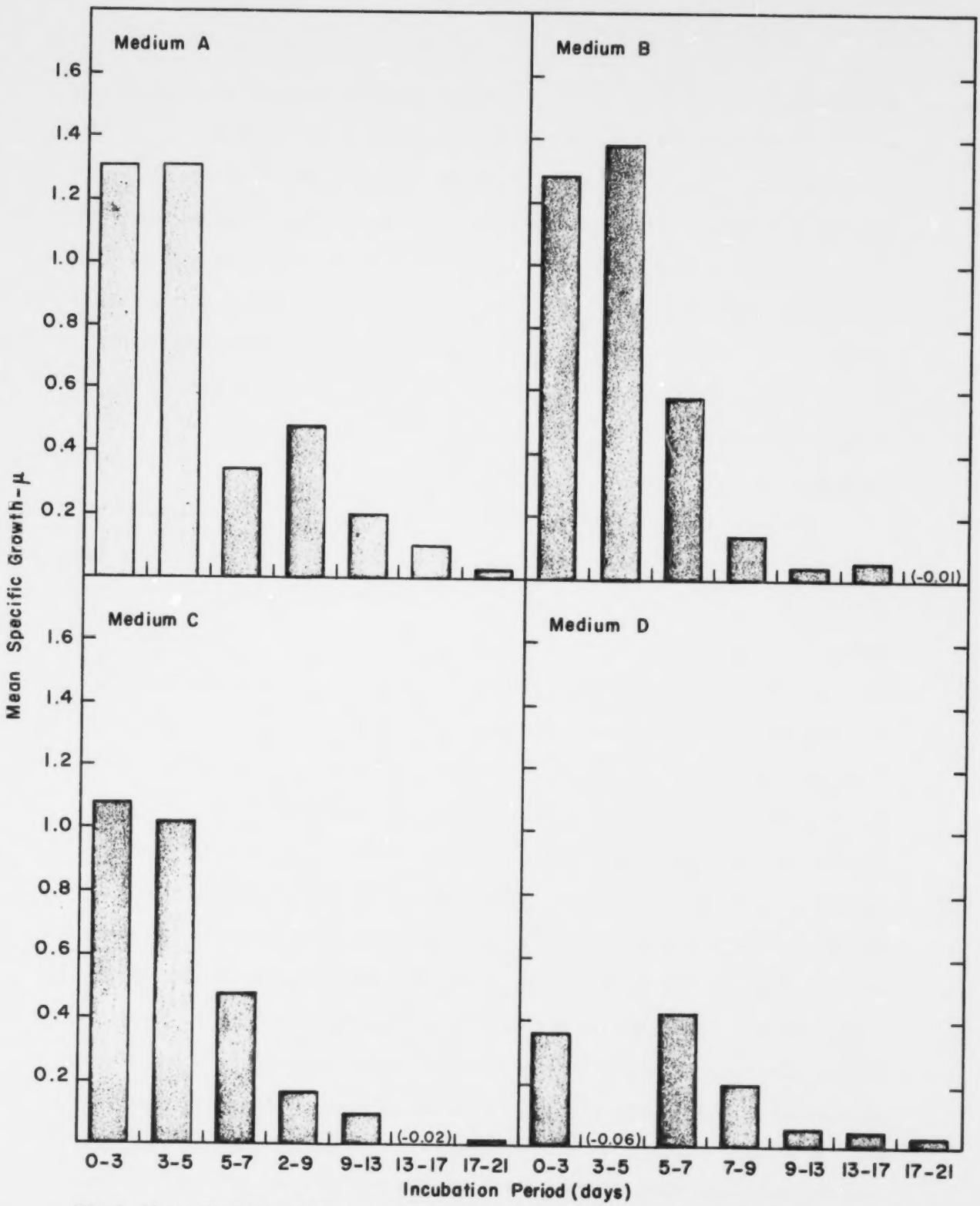

Fig. 6. Mean Specific growth rates for the indicated incubation periods, media A, B, C, D, (see table - for number of labs included in each average.)

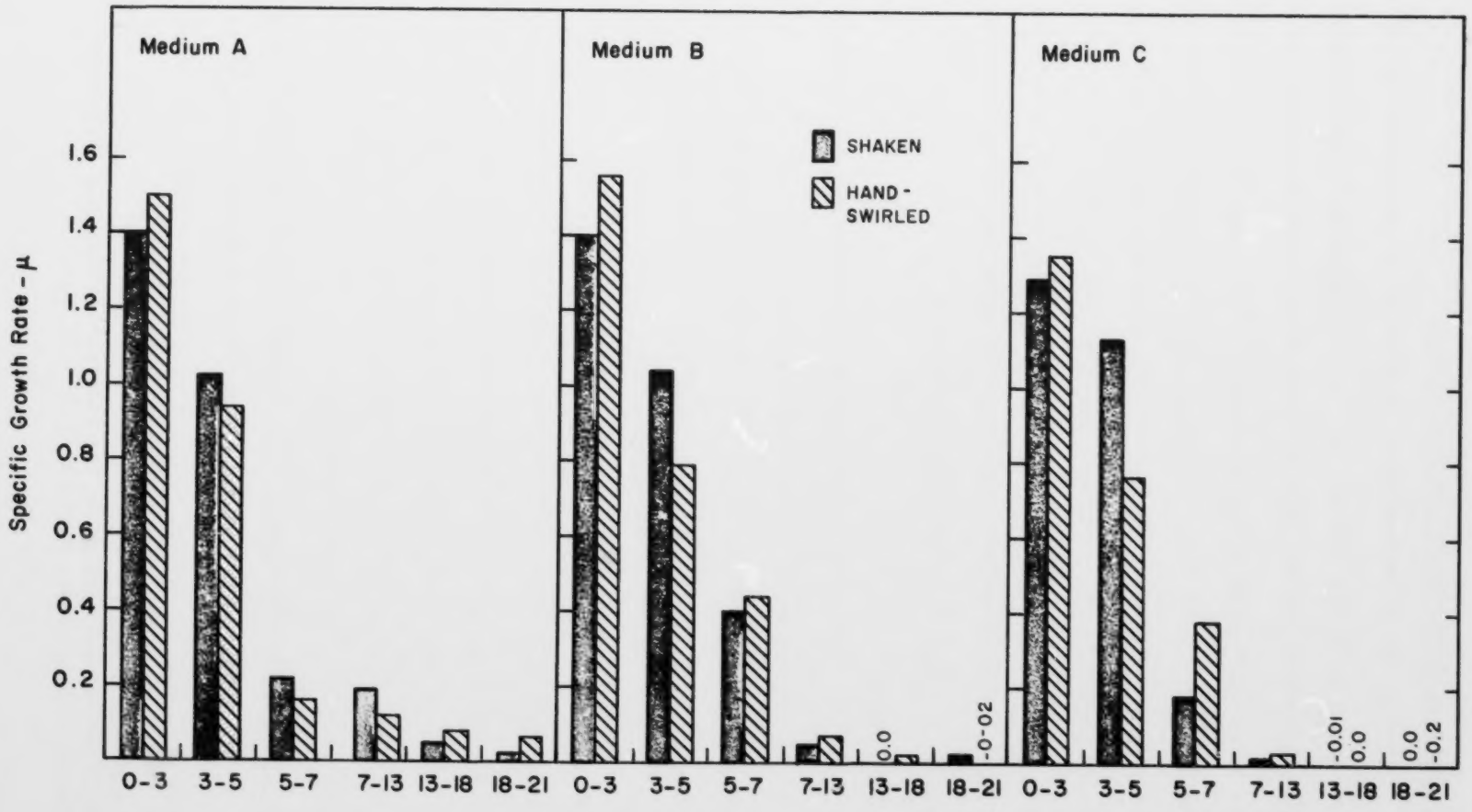

Fig. 7. Comparison of mean specific growth rates in shaker vs hand - swirled cultures, Lab No. 2, Corvallis cultures, UNC chemicals.

Comparison of Corvallis and Local Cultures

Several of the laboratories carried out a comparison test in which the growth response of the standard culture of Selenastrum capricornutum obtained in May, 1970 from the National Eutrophication Research Program was compared with their local culture of S. capricornutum. This comparison was made in the four mandatory media. The results arranged in Table 11 indicated that the reference algal culture, common to all laboratories, did not grow or respond in any manner that was significantly different than the local culture of the same species. In any growth period in which the comparison can be made, growth rates, while variable, showed no consistent pattern in which one of the cultures was significantly different than the other (Table 12).

Optional Media

In addition to the four mandatory media several optional media were suggested for comparison of growth response of the assay organism. These media were specifically different in that one or more of the known nutrient elements were removed or were replaced in amounts less than present in the NAAM medium.* The growth results, after 21 days, are shown in Table 13. The coefficient of variation of the mean between replicate samples was lowest in the complete medium and somewhat more variable, particularly between laboratories, in those media from which a critical element had been removed or replaced in somewhat lesser amounts. The diagram in Figure 8 is a comparison of the optional medium with the standard B medium. The effect of removal of critical nutrient elements or replacement at lower concentrations, in terms of growth response, is clearly indicated.

Correlations Between Weight Yield, Optical Density, Fluorescence and Total Carbon

The multi-laboratory investigation into the reproducibility of an assay procedure using the growth response of a specific species of algae also provided an opportunity to establish the relative levels of correlation between the basic

*The B medium was adopted as the reference medium and designated NAAM (New Algal Assay Medium)

Table 11

Comparison of Corvallis and Local Cultures: Means and Coefficient of Variation of the Mean in Cell Number, Log Cell Number, Dry Weight and Weight Per 10^6 Cells, at 21 Days

Type of Measurement	Medium	Laboratory 1				Laboratory 2				Laboratory 3			
		Corvallis		Local		Corvallis		Local		Corvallis		Local	
		$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var
cell no./ml	A	4.6[a]	13.5	6.0[a]	9.5	9.43[a]	11.6	---	---	1.1[a]	3.6	11.0[a]	5.1
						7.36[a]	3.4*	7.9[a]	5.4*				
	B	3.2[a]	5.8	3.4[a]	9.5	4.6[a]	3.7	---	---	5.5[a]	3.7	5.3[a]	6.6
						4.3[a]	3.2*	4.2[a]	5.1*				
	C	1.9[a]	6.3	2.0[a]	7.5	1.6[a]	3.9	---	---	2.0[a]	3.5	2.1[a]	2.6
						1.5[a]	41.8*	1.5[a]	4.2*				
	D	2.8[a]	17.0	1.6[a]	21.8	.0048[a]	72.7	---	---	.01[a]	37.5	.001[a]	0.0
						.0033[a]	10.5*	.01[a]	69.8*				
log cell no./ml	A	6.6	0.9	6.8	0.5	6.9	0.9	---	---	7.0	0.2	7.0	0.3
						6.9*	0.2*	6.9*	0.3*				
	B	6.5	0.4	6.5	0.6	6.7	0.2	---	---	6.7	0.3	6.7	0.04
						6.6*	0.2*	6.6*	0.3*				
	C	6.3	0.4	6.3	0.6	6.2	0.3	---	---	6.3	0.3	6.3	0.2
						6.2*	0.2*	6.2*	0.3*				
	D	5.4	1.9	5.1	2.6	3.3	7.3	---	---	3.1	3.9	3.0	0.0
						3.5*	1.4*	3.6*	7.0*				
dry weight mg/l	A	292.4	2.4	322.0	2.1	219.4	43.0	---	---	301.6	2.8	283.8	1.0
						181.4*	17.1*	178.9*	9.2*				
	B	168.4	2.9	171.0	5.4	127.1	2.7	---	---	137.8	3.1	134.8	2.1
						106.0*	2.7*	86.0*	17.5*				
	C	79.3	2.4	82.7	2.0	27.6	8.7	---	---	46.6	2.1	40.8	5.5
						32.5*	5.6*	30.0*	4.3*				
	D	44.1	17.9	33.6	10.6	---	---	---	---	---	---	---	---
weight/10^6 cell	A	6.7	13.2	5.5	5.9	2.4	4.8	---	---	2.7	5.7	2.5	4.7
						2.3*	5.7*	2.4*	6.1*				
	B	5.4	4.3	5.1	5.3	2.8	4.4	---	---	2.5	5.6	2.5	2.0
						2.5*	5.4*	2.1*	19.0*				
	C	4.2	8.0	4.3	9.6	1.7	8.6	---	---	2.3	4.7	1.9	7.6
						2.2*	7.8*	2.0*	5.7*				
	D	.24	51.9	.31	36.9	---	---	---	---	---	---	---	---

*Hand-swirled cultures
[a]These values are x10^6

Table 12

Comparison of Mean Specific Growth Rates of
Corvallis and Local Cultures, Media A, B, C and D, UNC Chemicals

Medium	Incubation Period (days)	Laboratory 1		Laboratory 2		Laboratory 3	
		Corvallis	Local	Corvallis	Local	Corvallis	Local
A	0-3	1.10	1.36	1.39	----	1.26	1.44
	3-5	1.11	0.86	1.03	----	1.52	0.85
	5-7	0.68	0.76	0.22	----	0.43	1.08
	7-9	0.13	0.09	----	----	0.26	0.28
	9-13	0.12	0.14	----	----	0.23	0.04
	13-17	0.13	0.15	----	----	0.06	0.07
	17-21	0.08	0.00	----	----	-0.01	0.04
B	0-3	1.32	1.49	1.39	----	1.27	1.48
	3-5	0.64	1.06	1.04	----	1.57	1.51
	5-7	1.01	0.66	0.40	----	0.57	0.44
	7-9	0.30	0.11	----	----	0.10	0.04
	9-13	0.08	0.00	----	----	0.05	0.01
	13-17	-0.01	-0.01	----	----	0.05	0.02
	17-21	-0.02	0.00	----	----	-0.01	0.00
C	0-3	1.22	1.43	1.29	----	1.32	1.51
	3-5	0.78	1.15	1.13	----	1.56	1.48
	5-7	0.01	0.54	0.18	----	0.16	0.00
	7-9	0.19	0.05	----	----	0.02	0.03
	9-13	0.01	-0.02	----	----	0.02	-0.01
	13-17	-0.04	0.01	----	----	0.01	0.02
	17-21	0.01	-0.03	----	----	0.01	0.02
D	0-3	0.48	0.39	----	----	0.00	0.00
	3-5	-0.56	0.25	----	----	0.00	0.00
	5-7	1.89	1.16	----	----	0.11	0.11
	7-9	-0.93	-1.04	----	----	0.07	0.19
	9-13	0.29	0.33	----	----	-0.03	-0.05
	13-17	0.49	0.57	----	----	-0.05	-0.10
	17-21	0.04	-0.16	----	----	0.07	0.00

Table 13

Comparisons of Optional Media, E - J, with Standard Medium B:
Means and Coefficient of Variation of the Mean in Cell Number Per Milliliter and
Log_{10} Cell Number Per Milliliter

		Medium													
	Lab No.	B		E no P		F no N		G no Fe		H only 6 µg P/l		I only 140 µg N/l		J only 1.1 µgFe/l	
		$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var	$\bar{x}$	%Var
cell no./ml	2	4.3×10^6	3.7	1.8×10^4	21.0	3.6×10^4	35.3	5.5×10^5	2.7	7.9×10^4	11.6	2.7×10^5	11.2	1.7×10^6	3.5
	3	5.5×10^6	3.7	1.4×10^3	17.5	2.1×10^5	6.5	3.5×10^5	14.9	6.1×10^4	8.7	4.3×10^5	5.0	1.4×10^6	12.3
	5	2.2×10^6	1.9	1.0×10^4	10.2	5.4×10^4	6.2	2.0×10^6	15.5	1.0×10^5	2.9	4.2×10^5	1.4	1.7×10^6	20.3
log_{10} cell no./ml	2*	6.63	0.2	4.20	2.3	4.47	3.1	5.74	0.2	4.89	1.0	5.43	1.0	6.25	0.2
	3	6.74	0.3	3.12	2.4	5.37	0.5	5.53	1.2	4.78	0.8	5.63	0.4	6.13	0.8
	5	6.33	0.1	3.99	1.1	4.74	0.6	6.28	1.1	5.02	0.3	5.63	0.1	6.21	1.3

*Lab #2 culture, media E - J, hand-swirled, whereas those from labs #3 and #5 were on a shaker (110 oscillations/minute).

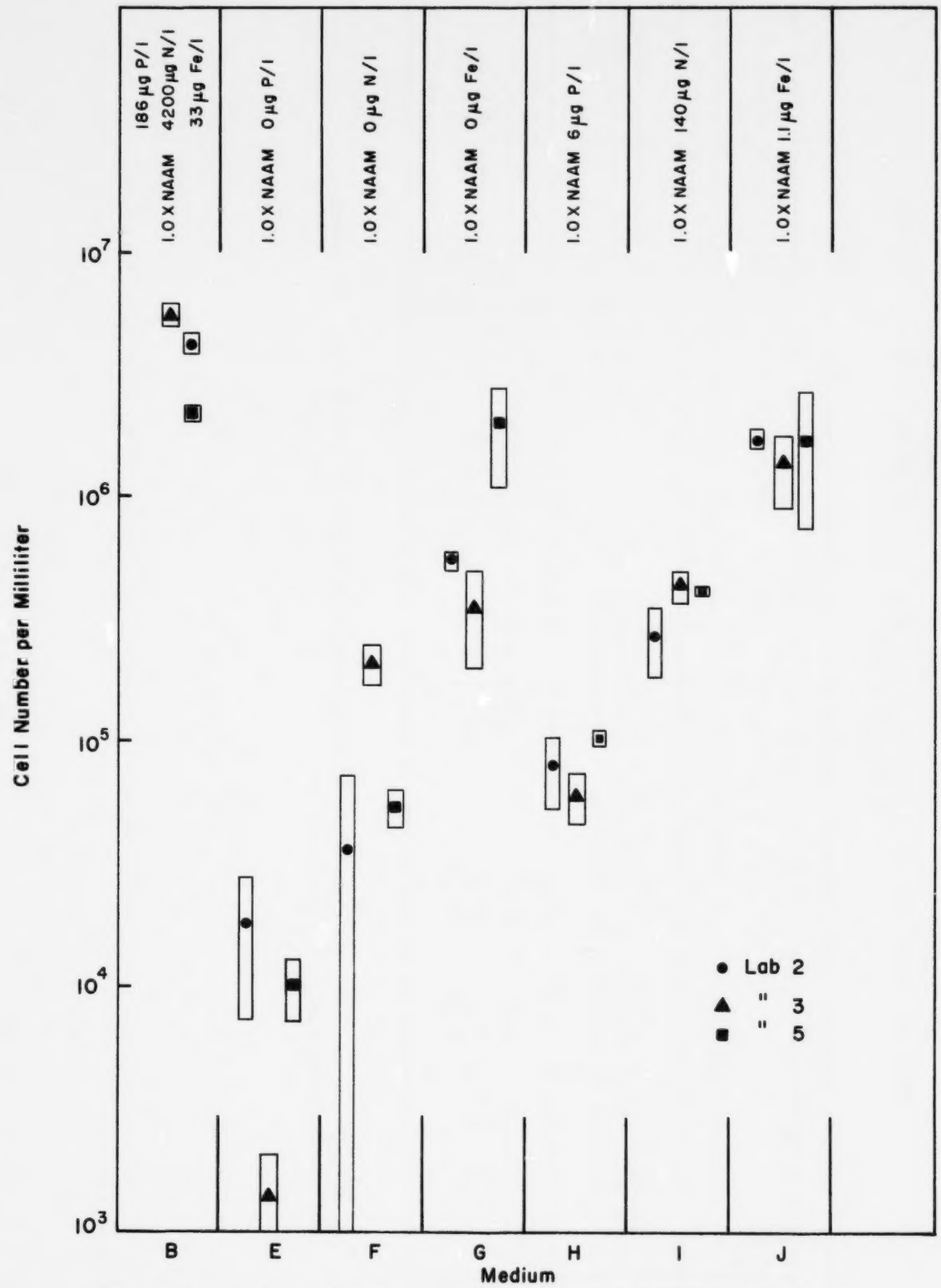

Fig. 8. Comparison of optional media E - J with standard medium B; mean cell number per milliliter and 95% confidence limits.

measurement of cell mass formed and more indirect techniques for following development of cell mass through the growth period. These included changes in optical density of the algal suspension, the fluorescence of chlorophyll in the multiplying cells and total carbon determination of washed algal suspensions. Data provided by Laboratory 3, which carried out many of the parallel measurements, lent themselves to an analysis to establish correlation coefficients between the parameters in question. A summation of this analysis is presented in Table 14.

Table 14

Correlation Between Algal Mass and
Other Measures of Algal Growth

Variables	Number of Samples	Correlation Coefficient	
			R^2
Wgt - Optical Density	20	.995	.99
Wgt - Total Carbon	20	.997	.99
Wgt - Fluorescence	20	.964	.93

If all samples, 20 in number, representing the results obtained from growth and other measurements in the four nutrient media are analyzed as a unit, it is indicated that correlation coefficients are extremely high for the relationship between weight and optical density, weight and total carbon and weight and fluorescence. However, an analysis of the same relationships in the individual media showed much lower levels of correlation. This peculiarity of this particular analysis is graphically shown in Figure 9, which depicts the very strong straight-line relationship between optical density and total carbon for the five replicates in each of the four media. It is clearly seen that a very straight line is generated by the clusters of points, but each cluster of points shows a rather low degree of linear distribution along the slope of the generated line rather than lying along the line. The

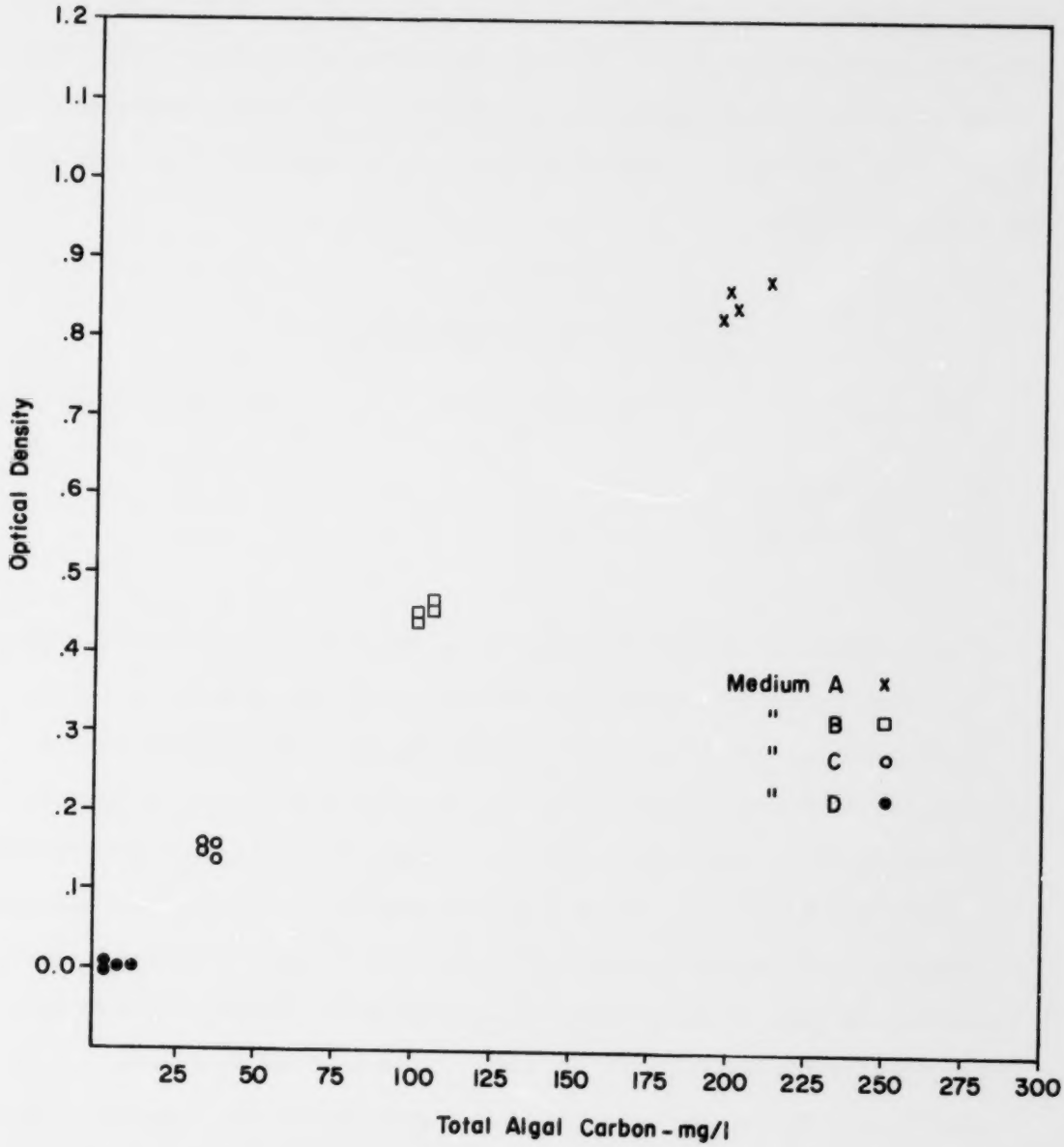

Fig. 9. The straight-line relationship between algal mass grown in media of various strengths and optical density of cell suspension. Note poor linear distribution of clustered points.

"tightness" of the clusters indicates a high degree of precision in the measurements. The low correlation in individual media indicates that comparatively poor correlation can be expected between replicates, but that in any series of media of varying nutrient strength, thus algal yield, correlation between the related techniques of measuring algal growth and mass are very good.

Regression Analysis - Nutrient Strength and Algal Yield

If an algal assay procedure is to be a measure of the growth response of some selected specie or species to known nutrient components so that a similar growth response might accurately reflect the presence of unknown growth promoting elements, it is essential to establish the degree of linearity of response to proportional quantities of known nutrient levels. The eight laboratories determined algal yield in the four mandatory media, representing media strengths of 3.3, 1.0, 0.33 and 0.033 of the unit medium strength. A regression anlaysis for each of the laboratories, individually and for all eight laboratories combined, was carried out.

The following function was fitted to the data of each lab for estimating dry weight:

$$\text{estimated dry wgt., } \hat{y} = a + b \text{ (strength of medium)}$$

(A preliminary test showed that a straight line would fit the data adequately for the range of media used.)

The analysis of regression for the eight laboratories as well as average values is summarized in Table 15, linear regression coefficients for dry weight as a function of medium strength. The variances and covariances of the coefficients are given in Table 16. The data of Tables 15 and 16 are

Table 15

Linear Regression Coefficients
for Dry Weight as a Function of Medium Strength

Lab No.	a = est'd intercept	s.d. of a	b = est'd slope	s.d. of b
1	57.925	6.480	77.780	3.711
2	32.296	7.097	49.944	3.520
3	35.051	8.692	78.873	4.311
4	19.726	9.164	52.665	5.248
5	28.478	12.292	120.958	6.096
6	32.755	9.164	68.523	5.248
7	52.064	8.130	58.837	5.233
8	50.646	15.711	99.278	7.401
Mean	38.618		75.857	
s.d.	13.336	10.00*	24.344	5.23*
c.v.	.3453		.3209	

underlying variance $s^2 = 921.482$

*pooled estimate of s.d.

Table 16

Variance-Covariance Matrices
For the Estimates

Lab		Variance-Covariance Matrix		
1	intercept, a slope, b	$\begin{bmatrix} .045567 = v_{11} & \\ -.017530 = v_{12} & .014941 = v_{22} \end{bmatrix}$		x 921.48*
2	a b	$\begin{bmatrix} .054658 & \\ -.020881 & .013443 \end{bmatrix}$		x 921.48
3	a b	$\begin{bmatrix} .081987 & \\ -.031322 & .020164 \end{bmatrix}$		x 921.48
4	a b	$\begin{bmatrix} .091134 & \\ -.035060 & .029883 \end{bmatrix}$		x 921.48
5	a b	$\begin{bmatrix} .16397 & \\ -.062444 & .040329 \end{bmatrix}$		x 921.48
6	a b	$\begin{bmatrix} .091134 & \\ -.035060 & .029883 \end{bmatrix}$		x 921.48
7	a b	$\begin{bmatrix} .071726 & \\ -.033268 & .029717 \end{bmatrix}$		x 921.48
8	a b	$\begin{bmatrix} .26787 & \\ -.10256 & .059438 \end{bmatrix}$		x 921.48

*this number represents the underlying variance, s^2

interpreted as follows. For Laboratory 1, for example, the estimated dry weight is

$$\hat{y} = 57.925 + 77.780 \text{ strength of medium}$$

$$\text{var}(\hat{y}) = s^2(v_{11} + 2v_{12}x + v_{22}x^2)$$

where $s^2 = 921.482$, $s \cong 30.36$

x = strength of medium

v_{11}, v_{12} and v_{22} come from Table 16

The essence of Table 15 is summarized in Figure 10, which shows dramatically how for each laboratory the intercepts are approximately equal, but the slopes vary somewhat.

This was further evaluated by carrying out a test of the hypothesis "all intercepts are equal". The test statistic is $F(7;194) = 2.601$, which is significant at the $\alpha = 0.014$ level. It was concluded from this test that there are significant differences among the intercepts as has been indicated in Figure 10.

A similar evaluation of slope functions was carried out by testing the hypothesis "all slopes are equal". The test statistic is $F(7;194) = 20.288$, which is significant at the 0.00001 level. It was concluded that there are significant differences among the slopes. It can be concluded from this evaluation that each laboratory participating in this interlab test had its own regression line. Also provided in Table 15 are the mean intercepts and slopes, their standard deviations and coefficients of variation. From these figures one can develop an idea of the variability of regressions from one lab to another. It can be stated that the variation in intercepts is primarily an underlying random variation as opposed to laboratory to laboratory variation. (Approximately 56% of the variability

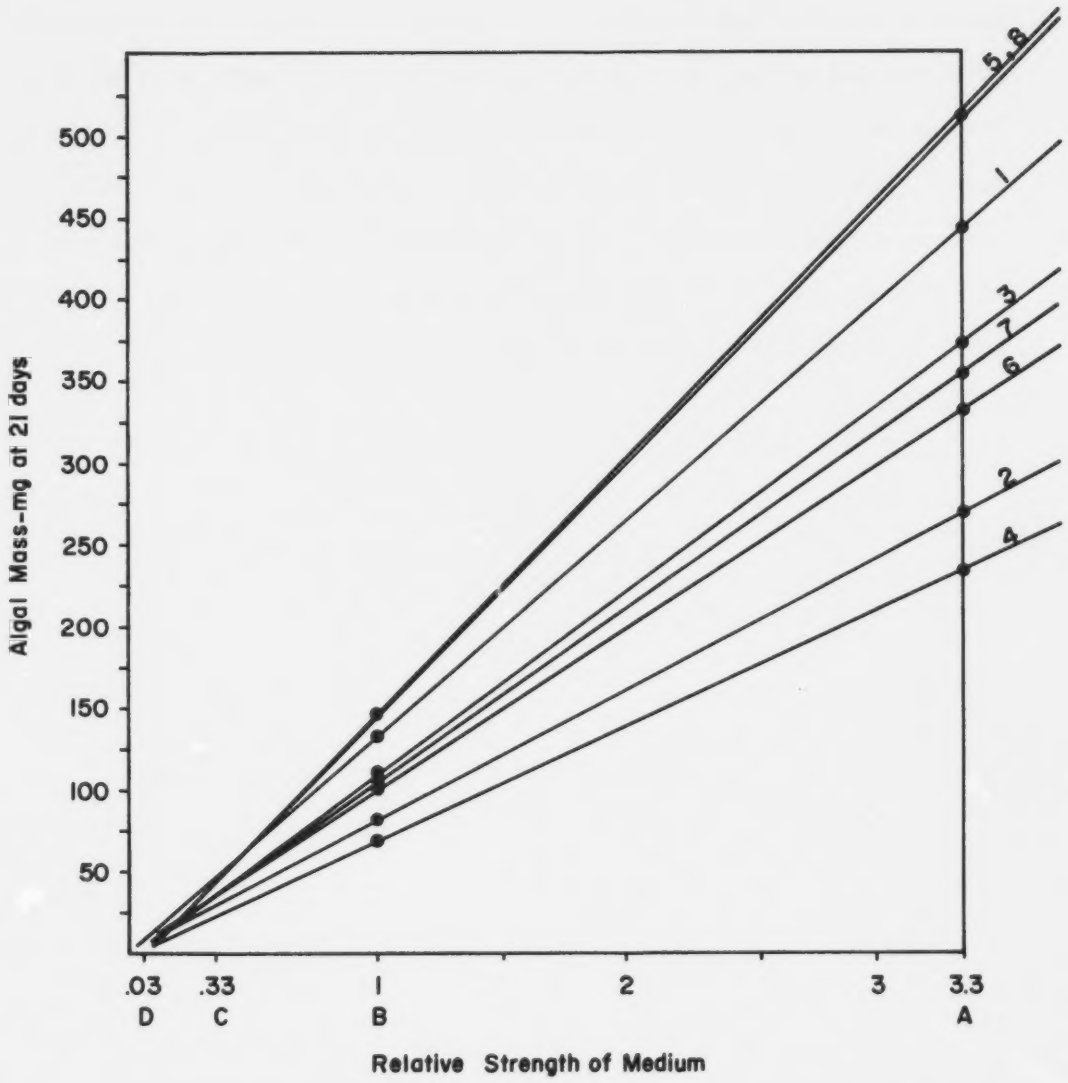

Fig. 10. Lines of regression relating strength of medium and algal mass. Number at end of line identifies laboratory.

is due to underlying random variation.) Most of the variability in slopes (approximately 95.4%) is due to lab-to-lab variability. This means, for example, that for the D medium the responses (dry weight) are fairly uniform from lab to lab, in most part due to the small growth response, but that there are quite significant differences in the responses to the stronger media. This would appear to be in conflict with the previous F-tests for equality of the intercepts. The interpretation is that even though the differences among intercepts are large enough to be measurable, they are relatively small when compared to differences among slopes.

Appendix A

Protocol For

Interlaboratory Precision Test

1. Scope

This test is a single attempt to test the precision of the PAAP Bottle Test among laboratories when several of the variables have been eliminated or minimized. Reagents from a common lot and an inoculum from a common source are prescribed.

2. Apparatus

2.1 Constant temperature room or equivalent incubator arrangement capable of providing temperature control at 24 ± 2°C.

2.2 Illumination with "cool white" fluorescent lighting to provide an intensity of 400 ± 20 ft. c. at the liquid level of the culture flasks. See Section 4.52.

2.3 Shaking apparatus for test culture flasks capable of 110 oscillations per minute.

2.4 Analytical balance capable of weighing 100 g. with a precision of ± 0.1 mg.

2.5 Oven, dry heat capable of temperature of 105 ± 1°C.

2.6 Culture flasks, Erlenmeyer, Pyrex-type.
500 ml. flasks shall contain 250 mls culture volume.

2.7 Culture flask closures, foam plugs.

3. Reagents

3.1 Algal Nutrient Medium

3.11 Macronutrients - Following salts in micrograms per liter of water distilled in glass from acid. Only reagents supplied by the

3.11 Macronutrients (Cont'd)

University of North Carolina, Chapel Hill, are to be used. (This is 30% of the concentration in the Feb. '69 PAAP medium with Na_2CO_3 having been replaced by $NaHCO_3$ and $Na_2EDTA \cdot 2H_2O$ having been reduced to 1 mg/l.)

Compound		Elements	
$NaNO_3$	25500 µg/l	N	4200 µg/l
K_2HPO_4	1044	P	186
$MgCl_2$	5700	Mg	2904
$MgSO_4 \cdot 7H_2O$	14700	S	1911
$CaCl_2 \cdot 2H_2O$	4410	Ca	1203
$NaHCO_3$	15000	K	468
		Na	11004

Note: No sterilization of the medium, either by filtration or by autoclaving, is preferred for this single test. If your counting method requires filtration, the trace metal-$FeCl_3$-EDTA mixture is added after filtration. With no filtration K_2HPO_4 should be added last to avoid iron precipitation. Stock solutions of individual salts may be made up in 1000 times the final concentration.

3.12 Trace Metals - Following salts in micrograms per liter of water distilled in glass from acid. Only the stock solution supplied by the University of North Carolina, Chapel Hill, is to be used.

3.12 Trace Metals (Cont'd)

Compound			
H_3BO_3	185.64 µg/l	B	33.0 µg/l
$MnCl_2$	264.27	Mn	114.0
$ZnCl_2$	32.70	Zn	15.0
$CoCl_2$	0.78	Co	.35
$CuCl_2$	0.009	Cu	.003
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3$	96.0	Fe	33.0
$Na_2EDTA \cdot 2H_2O$			

Note: The trace metals, $FeCl_3$ and EDTA, will be combined in a single stock mix at 1000 times the final concentrations.

3.13 Optional Media - Prepared from each laboratory's own stock chemicals for replicate experiment to compare with chemicals supplied by University of North Carolina, Chapel Hill.

4. Procedure

4.1 Preparation of test waters - Test waters will consist of four concentrations of the nutrient medium specified in Section 3.1. All test waters will be run with 5 replications.

4.11 Mandatory Test Waters

A - 3.33 x Nutrient Medium specified in Section 3.1.

B - 1.0 x " "

C - 0.33 x " "

D - 0.033 x " "

4.12 Suggested Optional Test Waters

E - 1.0 x	Nutrient Medium		without P*
F - 1.0 x	"	"	without N
G - 1.0 x	"	"	without Fe
H - 1.0 x	"	"	with only 6 µg P/l**
I - 1.0 x	"	"	with only 140 µg N/l
J - 1.0 x	"	"	with only 1.1 µg Fe/l

*add 892 µg KCL to provide K
**add 802 µg KCL to provide K

4.13 Additional Optional Test Waters

1.0 x nutrient medium omitting in separate cultures Mg, S, Ca, K and trace metals.

4.2 Production of Inoculum - The inoculum species to be used in this test will be a special standard culture of Selanstrum capricornutum supplied by the National Eutrophication Research Program, Corvallis, Oregon. Upon receipt of the inoculum species, a portion should be transferred to the algal nutrient medium specified in Section 3.1. (Example: 1 ml of inoculum to 250 ml of algal nutrient medium in 500 ml Erlenmeyer flask.) This transfer from the special standard culture is repeated daily, if desired, for one week. On the eighth day the transfer will be made from the new seven-day old culture instead of the original inoculum. The volume of this transfer is calculated to result in a concentration of 10^3 cells/ml in the new culture flask. This procedure will be repeated thereafter for each desired working day; only seven-day old cultures will be used for inocula. These stock cultures should be incubated under the same conditions as prescribed for the test flasks.

4.3 Preparation of Inoculum - After no less than three and no more than seven weekly transfers, the cultures will be used to inoculate the test waters specified in Section 4.1.

Cells from a seven-day old stock culture are centrifuged and the supernatant discarded. The sedimented cells are resuspended in an appropriate volume of a 15000 $\mu g/l$ $NaHCO_3$ solution and again centrifuged. The sedimented cells are then resuspended in the $NaHCO_3$ solution, counted and a portion is pipetted into each test water.* The volume of the transfer is calculated to result in a concentration of 10^3 cells/ml in the test flasks. (Example: 5×10^5 cells/ml in stock culture requires a 0.2 ml transfer per 100 ml test water.) Transfer should not exceed 1 ml per 100 ml (a minimum 1×10^5 cells/ml in stock culture is required).

*If preferred, a final volume can be calculated and made up with the $NaHCO_3$ solution such that each 0.1 ml (or any convenient transfer volume $\leq$ 1 ml) contains 250,000 cells.

4.31 Comparison of Cultures - Prepare duplicate culture of Selenastrum from existing stock in each participant's own laboratory for comparison with culture supplied by NERP.

4.4 Preparation of Test Flasks - Five replicate flasks will be prepared for each test water variable. Each 500 ml flask will be pre-rinsed with approximately 60 ml of the test water to be added to the flask. 250 ml of the test waters are added to the test flasks, the test waters are inoculated at 10^3 cells/ml and the flasks are stoppered with foam plugs.

4.5 Incubation - Test flask cultures are incubated at the following controlled environmental conditions.

4.51 Temperature - 24° ± 2°C. Measurements are to be made at 3 random locations on the shaker platform and recorded on the standard report form daily.

4.52 Illumination - Continuous "cool white" fluorescent lighting to provide 400 ± 20 ft - c measured outside the flask at the liquid level. For this test, correction factors will be used based on the comparison readings made at the Corvallis meeting. (See Summary of PAAP Evaluation Meeting, Item 5.) The Weston units were the agreed upon standard meters. Therefore, to establish 400 ft - c, adjust lights to give a reading on your meter equal to 0.67 times your reading at Corvallis. This admittedly is not theoretically sound because of the differing spectral sensitivities and optical geometries of the meters, but it seems a practical compromise until a more sound standardization procedure is established.

4.53 Shaking - Continuous gyrotary, 110 oscillations per minute.

4.6 Observations

Sample Volume - Do not take more than a total of 50 ml from each test flask during the first 20 days; i.e., 5-10 [illegible] sampling time. If greater sample volume is desired, [illegible] flasks which can be terminated at time of sampling.

4.62 Cell Count Measurement - Any method of count (i.e., cell number/ml) determination is applicable including direct microscopic counting (hemocytometer) or the use of electronic particle counters. Counts will be made at least every other day during log-phase growth, but may be less frequent after the log phase. Preferred counting days are: 3, 5, 7, 9, 13, 17 and 21.

4.63 Gravimetric Measurement - Dry weight of algal mass is measured at the end of the 21 day test.

A suitable portion of algal suspension is centrifuged. The supernatant is discarded, the sedimented cells are resuspended in distilled water, transferred to tared crucibles or aluminum cups*, dried overnight in a hot air oven at 105° C., cooled in a desiccator over Drierite for 1 hour, and weighed.

*Aluminum cups should be pretreated before taring by adding approximately 15 mls of distilled water and drying 105° C. in the oven. This pretreatment should be repeated for a total of three times; then the cups can be tared accurately. A continuous flow centrifuge may be used if available.

4.64 pH Measurements - pH measurements will be made on all flasks of treatments A, B, C, and D (Section 4.11) at least for days 3, 9, and 21 and recorded on the standard report form.

4.65 Other measurements may be made as desired. CAUTION: See 4.61

5. Reports

Data shall be entered on the standard report form and returned to the University of North Carolina, Chapel Hill, for statistical analysis.

Appendix B

SOME STATISTICAL CONSIDERATIONS IN THE ALGAL ASSAY

Log Cell Count vs Actual Cell Count

There are three primary statistical reasons for analyzing log cell count in addition to (or instead of) actual cell counts. First, it is very obvious that the variance or standard deviation among replicate flasks is much more homogeneous for log cell count than for actual cell count. That is, the differences among standard deviations for flasks under different experimental combinations are much smaller for log cell count than for actual cell count. This has several advantages. The assumption that the (unknown) population standard deviations are all identical is plausible with log cell count, but not with actual cell count, which allows us to use pooled estimates of variance, giving us more powerful tests and better estimates of variances. This homogeneity also means that the data come closer to satisfying the assumptions underlying the statistical comparisons of treatments (e.g., differences between shaking and swirling).

A second reason for using the log transformation is that the variation among log cell counts from replicate flasks tends to have a more "normal" distribution than does the variation among actual cell counts from replicate flasks. A third reason, perhaps the most compelling, involves the correlation between means and standard deviations. It is quite obvious that the treatment combinations which yield large numbers of algae have large flask-to-flask variability among replicate flasks. This correlation between mean and standard deviation is in the neighborhood of 0.8, which is quite large.

For biological data which span several orders of magnitude, the log transformation often corrects all three problems: it produces homogeneous

variances, the logarithms of the data appear to have a more-nearly normal distribution, and the correlation between means and standard deviations often disappears. In this case the log transformation goes far toward producing homogeneity and normality, but there is a negative correlation between means and standard deviations of the log data.

Practically speaking, it is very interesting to observe that the precision, as measured by the coefficient of variation, c.v. = $\frac{\sigma_{\bar{x}}}{\bar{x}}$, is roughly an order of magnitude better for log cell count than for actual cell count. This result is probably due to the fact that the log transformation greatly reduces the effect (on the standard deviation) of one or two flasks which differ greatly from the others in a replicate set. If the log transformation reduces the spread of the distribution of observations relative to the mean, i.e., if it decreases the c.v. so drastically, then it certainly should be used.

Along a more philosophical line, one must consider which scale of measurement is more appropriate: actual cell count or log cell count. The statistician cannot and should not make this determination. In considering this problem one should take into account the wide range of cell counts which occur in this problem. For example, in these experiments counts varied from 1000 = 1×10^3 to about 2×10^7 = 20,000,000. Suppose the average numbers of cells under two different conditions were 1000 and 2000, a difference of 1000. Does a difference of 1000 cells here have the same significance as the difference of 1000 cells between averages of 20,001,000 and 20,000,000? Is a difference in the 5th place as meaningful as a difference in the 1st digit? Is the answer is "no" then a log scale is strongly

suggested as a more meaningful scale of measurement than an arithmetic scale.

Outliers

An "outlier" among replicate observations is one whose deviation from the mean is far greater than the rest in absolute value and perhaps lies three or four standard deviations or further from the mean. The outlier is a peculiarity and indicates a data point which is not at all typical of the rest of the data. It follows that an outlier should be submitted to particularly careful examination to see if the reason for its peculiarity can be determined.

Rules have been proposed for rejecting outliers, i.e., for deciding to remove the observation(s) from the data, after which the data are reanalyzed with these observations. (Such a procedure is given below.) Automatic rejection of outliers is not always a very wise procedure. Sometimes the outlier is providing information which other data points cannot due to the fact that it arises from an unusual combination of circumstances which may be of vital interest and requires further investigation rather than rejection. As a general rule outliers should not be rejected out of hand unless they can be traced to causes such as errors in recording observations or in setting up apparatus. Otherwise careful investigation is in order. [The above was adapted from section 3.8 of Applied Regression Analysis by N. R. Draper and H. Smith, John Wiley & Sons, 1968.]

Test for Discarding Outliers.

When a definite cause cannot be located for an outlier the following test may be applied.

1. Rank order the data in the group containing the outlier (all observations in the group are supposedly treated alike):

$$X_1 \leq X_2 \leq \cdots \leq X_n.$$

2. Compute the appropriate criterion:

If X_1 is the outlier: $c = \dfrac{X_2 - X_1}{X_n - X_1}$

If X_n is the outlier: $c = \dfrac{X_n - X_{n-1}}{X_n - X_1}$

3. If c exceeds the critical value opposite "n" in the following table, reject the outlier.

n	critical values $\alpha = .05$	$\alpha = .01$
3	.941	.988
4	.765	.889
5	.642	.780
6	.560	.698
7	.507	.637

Example. Suppose the following replicate observations are made: 9.8, 4.7, 8.4, 8.0, 8.4, 7.9. The value 4.7 suspect. We rank order the data:

X_1	X_2	X_3	X_4	X_5	X_6	
4.7	7.9	8.0	8.4	8.4	9.8	n = 6

The criterion is

$$c = \frac{X_2 - X_1}{X_n - X_1} = \frac{7.9-4.7}{9.8-4.7} = \frac{3.2}{5.1} = .63$$

Since $n = 6$, this value is significant at the $\alpha = .05$ level ($0.63 > 0.560$) but not at the $\alpha = .01$ level ($0.63 < 0.698$).

The experimenter who is willing to discard 5% of all his good data would discard the observation 4.7 as an outlier. The experimenter who is willing to discard only 1% of all his good data would keep the observation, unless, of course, he could find an experimental reason for discarding it. [These percentages are based on the assumption of normality.]

4. If there are two suspected outliers (say X_1 and X_n or X_1 and X_2) the test may be repeated; apply it to the "worst" outlier first.

5. Note that the regular use of this procedure will result in discarding 5% (if $\alpha = .05$) or 1% of all one's <u>good</u> (valid) observations.

Appendix C

A GUIDE TO THE DESIGN OF COMPARATIVE ALGAL ASSAY EXPERIMENTS AND SELECTION OF REPLICATE SIZE

Introduction

The goal of this appendix is to present some guides to the design of very simple comparative experiments, with particular reference to algal assay experiments. For any experiment of more than modest size or cost the experimenter is strongly urged to consult a statistician, particularly one with an interest in experimental designs, for help in designing the experiment.

Comparative Experiments vs. Descriptive Experiments

The text of this report clearly illustrates one aspect of algal assays: the results from one laboratory are in much closer agreement than the results from several laboratories. This effect may be the result of different experimental setups, different personnel, or other factors, but regardless of the source of the differences the conclusion to be drawn is that these systematic differences are so large that it is not practical to compare, for instance, a cell count from one lab to a cell count from another lab, or a dry weight determination from one lab with a dry weight determination from another lab. If one were to make such a comparison most of the differences in the results would be due to the difference between laboratories, not the difference between different treatments.

Because of these large lab-to-lab differences it is not practical to make a precise estimate of the number of cells that might grow on a medium such as Medium B, after 21 days under the standard conditions described in the protocol. However, the reasonably good agreement among replicate flasks

in any one lab indicates that for a small enough group of flasks the systematic effects apply evenly so that it is possible to get a quite accurate determination of the difference in the effect of two treatments (two media, for example).

It is important to notice the distinction between (a) estimating the difference between the effects of two treatments and (b) estimating the total number of cells (or dry weight) which will grow under certain "standard" conditions. In case (b) the mere fact that labs are different, have different calibration problems, different growth environments, and so on, means there will be great systematic effects associated with each lab. However, if numbers of cells in two flasks from the same lab, with identical environments except for treatment differences, are considered it is clear that when the difference is examined between the observed numbers of cells in the two flasks all the systematic environmental effects cancel, leaving only the difference due to difference in treatment effects and due to random measurement errors and random environmental effects. A comparative experiment is one in which one observes such differences, i.e., one compares the effects of different treatments. A descriptive experiment is one in which one estimates a particular chemical, physical, or biological constant, as, for example, Avogadro's number or the number of cells which will grow on Medium B in 21 days under "standard" conditions.

Algal assays, if properly designed and executed, can lead to quite precise comparative experiments, but descriptive algal assays have been seen to have poor precision. In this guide we consider only comparative experiments.

Basic Ideas of Comparative Experiments and Testing Hypotheses

Consider for a moment the basic purpose of a comparative experiment for comparing two different "treatments", e.g., two types of medium nutrients. The usual purpose is to determine whether one treatment has the effect of

yielding more algal growth than the other, and, if so, which one and by how much? The phrase "one has the effect of yielding more algal growth than the other," has to be precisely defined and to do this some simple mathematical formulas will be introduced. Suppose two treatments are to be compared, designated #1 and #2, as indicated above. Also suppose that under certain "very standard" conditions, if no errors were made, #1 would yield exactly m_1 algae cells at the end of the growth period and #2 would yield m_2 cells. No laboratory can match the "very standard" conditions exactly, so let us assume that being in the lab under consideration has the effect of adding b cells to the total. Consider a pair of flasks treated as identically as possible, one getting each treatment; by virtue of the fact that the flasks are placed in a certain environment within the lab the final numbers of cells will differ from $m_1 + b$ and $m_2 + b$; let the environmental effect, for <u>this</u> pair of flasks within this lab, be c cells. If no errors were made the flasks would end up with $m_1 + b + c$ and $m_2 + b + c$ cells respectively. However, small errors (hopefully random) are made in inoculating, shaking, counting, etc., so that the final yields, the final numbers of cells counted are y_1 and y_2: thus

$$y_1 = m_1 + b + c + e_1 \qquad \text{for \#1}$$
$$y_2 = m_2 + b + c + e_2 \qquad \text{for \#2}$$

where e_1 and e_2 represent the sums of all the effects of the various errors. Note that b (effect due to lab) and c (effect due to environment within lab) are systematic, but are identical for each flask. Note that all the systematic errors disappear when we consider the difference:

$$d = y_1 - y_2 = (m_1 - m_2) + (e_1 - e_2).$$

Now we have a precise way of asking "whether one treatment has the effect of

yielding more algal growth than the other". By this we mean does $m_1 = m_2$? For convenience we define one more symbol:

$$\delta = m_1 - m_2$$

Our question now is: Does $\delta = 0$?

It is clear after a moment's reflection that δ is never exactly zero; no two different treatments, even under ideal conditions, would yield exactly the same algal growth; there will always be a slight difference. The question must be rephrased, "Is the difference between the effects of the treatments detectable in a reasonably-sized experiment?" Is δ large enough (or, if $\delta < 0$, small enough) compared to the size of the random errors e_1 and e_2 so that we will always get a value of d too big to be explained as random error?

Before considering the answer to that question, consider the random errors e_1 and e_2. We assume these errors really are random, not systematic, and therefore will tend to "cancel each other out". Morever, we will take several pairs of flasks and hope the random errors "average out" to be very close to zero so that the average difference, call it $\bar{d}$, will be close to $m_1 - m_2$. If the errors really are random and are not systematic, they will average out. This is a very important point and will be discussed below under the topic of "randomization".

Hypothesis Testing

Our formal statistical procedure will be based on the following reasoning process. We are interested in proving that $\delta \neq 0$. Let us assume, or hypothesize, that $\delta = 0$. If the data contain strong evidence to the contrary we will reject the assumption that $\delta = 0$ and conclude $\delta \neq 0$. If the evidence from the data is not strong enough we will say we do not have enough evidence to reject the

assumption that $\delta = 0$. (Note that we do not conclude $\delta = 0$; we simply cannot reject the assumption.)

There is a well-known analogous procedure which will help make this clear. In principle, at least, when a man is charged with a crime in the U. S. it is initially assumed by the judge and jury that the man is innocent. (This is analogous to our initial assumption that $\delta = 0$.) After the evidence is presented, if it is sufficiently convincing, the defendent is declared guilty, i.e., the initial assumption of innocence is rejected (the assumption $\delta = 0$ is rejected). However, if the evidence is not strong enough, the judge may dismiss the charges, i.e., not reject the initial assumption. The analogy fails if the defendent is acquitted; we rarely ever acquit δ, i.e., actually find that there is sufficient evidence to conclude that $\delta = 0$.

Just as the court system makes errors, because our data contain random errors we occasionally come to the wrong conclusion. There are two types of errors we can make:

Type I: Conclude $\delta \neq 0$ when in truth $\delta = 0$.
Type II: Fail to conclude $\delta \neq 0$ when in fact $\delta \neq 0$.

We always set the probability of a Type I error:

The "significance level" of a test is the probability of a Type I error (denoted with Greek letter α).

By careful design of the experiment we can also choose the probability of a Type II error.

Note that in any particular experiment we will never be certain whether we have drawn an erroneous conclusion, but by always working with experiments with low probabilities of these errors over a long period of time the proportions of errors made will closely match those low probabilities.

Computing the Number of Replicates for a Paired t-Test

With the introductory information above and some results from the Interlaboratory Precision Test we are now prepared to discuss the necessary size of an experiment in which two treatments are being compared with paired flasks as discussed above. The basic question is: How many pairs of flasks are needed? The answer to this question will be based on the answers to the following questions and some simple arithmetic.

Question 1. What significance level will be used? Recall that the significance level is the probability of concluding $m_1 - m_2 \neq 0$ (i.e., that there is a difference between treatments) when, in fact, there is no difference. Typical values of the significance level are $\alpha = 1/20$ (one error for each 20 comparisons, on the average), $\alpha = 1/100$, or $\alpha = 0.025$. Choose your own α depending on the percentage of the time you want to tell a lie, i.e., wrongly report there is a significant difference. (The tables attached [C-1] work only for $\alpha = .05$ or $\alpha = .01$.)

Question 2. What is the smallest difference, $\delta = m_1 - m_2$, which must be detected? Is a difference of 100 cells, out of a million, important enough to detect? Or should the smallest detectable difference be 10,000 cells, or 100,000? This question can be rephrased as: Statistical considerations aside, what is the smallest difference that is in fact "significant"? (You may change your mind after computing sample size!)

Question 3. Assuming the difference is exactly equal to the difference specified as the answer to question 2, i.e., the smallest difference which must be detected, with what probability must the experiment detect this difference (reject the assumption of a zero difference)? That is, do you want a 99% chance of detecting a difference of that size? a 90% chance? a 50-50 chance? Clearly

if the true difference is larger than the one specified in Question 2 the probability of detecting it will be greater than the answer to this question, and vice versa.

Question 4. What is the standard deviation of an individual error (e_1 or e_2) or observation? The answer to this question can be obtained from the results of the Interlab Precision Test. Find in Table C-2 the particular medium, type of measurement, and lab which most closely match the environment and treatments of the new experiment. Find the estimate of the standard deviation (not the standard error) for that combination; it is the approximate answer to this question. If no lab clearly matches, use the average standard deviation over labs. To be conservative use the largest value from the lab with the largest standard deviation for the same type of measurement, medium, etc.

Question 5. If $\delta \neq 0$, i.e., $m_1 \neq m_2$, is the sign of δ known? That is, if $m_1 \neq m_2$ is it definitely known whether $m_1 > m_2$? If so, the test we will perform after the data are collected will be a "one-tail paired t-test"; if it is not known in advance whether $m_1 \geq m_2$ the test will be a "two-tailed paired t-test".

Computing the Number of Flask Pairs

For ease in writing formulas and looking up values in tables define the following symbols for the answers to the questions above:

	Question	Symbol
1.	Level of significance	α
2.	Smallest detectable or significant δ:	δ_o
3.	Probability of declaring $\delta \neq 0$ (if $\delta=\delta_o$): (i.e., β = probability of type II error)	$1-\beta$
4.	Standard deviation of an observation:	s

Instructions:

1. If the answer to Question 5 is YES, use the "One-Tail Test Tables" below; otherwise use the "Two-Tail Test Tables
2. Compute $d = 0.7071\ \delta_o/s$

3. For the specified values of α, $1-\beta$, and d, find the value of "u" from the correct table (see instruction #1).
4. If $\alpha = .01$ $\quad n = u + 2$
   If $\alpha = .05$ $\quad n = u + 1$

The value of n is the number of pairs of flasks which must be used. If this number seems too small check your values of $1-\beta$, δ_o, and s. If the number seems too large, check your answers to the questions for reasonableness, and check your arithmetic. If the number still seems too large the experiment may be unrealistic: consult a statistician.

Warnings

The guidelines above lead to an experiment for comparing two "treatments" to determine which, if any, yields a greater algal growth. The power and validity of the experiment depend critically on the pairing of the flasks. Both flasks of any one pair must have an identical environment insofar as is experimentally possible. Both flasks in a pair should be "measured" (counted, weighed, etc.) as identically as possible. The Interlab Precision Test demonstrated that even under carefully controlled conditions systematic errors can enter an experiment. In the experiment described above it does not matter if different pairs receive different environments; that is expected. It is extremely crucial that the two flasks in any pair be as identical as possible, except, of course, for the difference in treatments.

Randomization

One powerful technique for eliminating systematic effects between the flasks in a pair is to randomly make all decisions which might affect environments. Randomly choose (by coin flip, for example) which treatment gets which flask, which flask takes which position on the incubation table, which is inoculated first, which is counted first, and so on. A different randomization is

used for each pair. The randomization is important for two reasons: first, it eliminates the possibility of unknown systematic effects (not in any one pair, but on the average); secondly, it helps assure the validity of the statistical analysis by making tests into so-called "randomization tests".

Analysis of the Data

Data arising from an experiment designed along the guidelines given above are easily analyzed. First, for each pair of flasks compute the difference between the two measurements (always in the same order, say treatment #1 measurement - #2 measurement). For the i-th flask call the result d_i. There will be n such values. Compute the mean and standard deviation of the d_i's; call these $\bar{d}$ and s_d. The t-test statistic is given by

$$t = \frac{\bar{d}}{s_d/\sqrt{n}} \qquad \text{with n-1 degrees of freedom.}$$

This t-value can be referred to an appropriate (one-tail or two-tail) table of critical values. $\bar{d}$ is the average difference, or the differences of the averages, of the two treatments.

Note: If any flask of a pair should for some reason either not be available for measurement or should receive a different environment from its mate, ignore both members of the pair when analyzing the data.

More Than Two Treatments

Most experiments are set up to compare more than two "treatments". Sometimes there are several different types of "treatments", say different media, different chemical additions, etc. The guidelines above can be extended to more than two treatments by using triplets for three different treatments instead of pairs, or quadruplicates for four different treatments, and so on. The limit on the number of different treatments is set by the limit on the

number of flasks which can be given identical environmental conditions. The critical aspect is that each flask in any given pair, triplet, etc., must receive exactly the same environment as every other flask in the "replicate". ("Replicate" and "block" are statistician's terms to describe pairs, triplets, etc., of flasks with the same environment but different treatments.) If the number of treatments is so large that environmental conditions are not identical for all flasks in a replicate then either the number of treatments should be decreased or a statistician should be consulted. There are special experimental designs for such situations.

An experiment as described above with more than two treatments is called a Randomized Complete Block experiment. Standard introductory statistical texts contain instructions for the statistical analysis of such experiments. In addition, most computer facilities have standard programs for such analyses. The use of such a program is probably the least expensive, most efficient method of getting the data analyzed. Questions about the interpretation should be referred to a statistician.

Summary

A major conclusion to be drawn from the Interlab Precision Test is that descriptive algal assay experiments have relatively poor precision: it is not practical to produce precise estimates of the biomass which would be produced under a set of "ideal" conditions. The loss of precision is a result of rather large systematic differences among labs. Moreover, within any one lab it is not possible to estimate the accuracy of experimental results because the same systematic error is manifest in all measurements within a lab.

However, a second major conclusion is that comparative algal assays--experiments for estimating the difference between the biomasses which would be produced under two sets of ideal conditions--in comparison are quite precise. That is, small differences in strengths of media can be detected.

TABLE C-1. AID IN COMPUTING SAMPLE SIZES REQUIRED TO DETECT PRESCRIBED DIFFERENCES BETWEEN AVERAGES

Notation:

- α Significance level of the test
- δ_o Smallest detectable or significant difference
- $1-\beta$ Probability of declaring $\delta \neq 0$ if $\delta = \delta_o$.
- s Sample estimate of the standard deviation of an observation
- d $= 0.7071\ \delta_o\ /\ s$

ONE TAIL TEST TABLES:

For $\alpha = .01$ add 2 to the tabled value to get the number of pairs (replicates); for $\alpha = .05$ add 1 to the tabled value to get the number of pairs.

$\alpha = .01$

d \ 1-β	.50	.60	.70	.80	.90	.95	.99
.1	542	666	813	1004	1302	1578	2165
.2	136	167	204	251	326	395	542
.4	34	42	51	63	82	99	136
.6	16	19	23	28	37	44	61
.8	9	11	13	16	21	25	34
1.0	6	7	9	11	14	16	22
1.2	4	5	6	7	10	11	16
1.4	3	4	5	6	7	9	12
1.6	3	3	4	4	6	7	9
1.8	2	3	3	4	5	5	7
2.0	2	2	3	3	4	4	6
3.0	1	1	1	2	2	2	3

$\alpha = .05$

d \ 1-β	.50	.60	.70	.80	.90	.95	.99
.1	271	361	471	619	857	1083	1578
.2	68	91	118	155	215	271	395
.4	17	23	30	39	54	68	99
.6	8	11	14	18	24	31	44
.8	5	6	8	10	14	17	25
1.0	3	4	5	7	9	11	16
1.2	2	3	4	5	6	8	11
1.4	2	2	3	4	5	6	9
1.6	2	2	2	3	4	5	7
1.8	1	2	2	2	3	4	5
2.0	1	1	2	2	3	3	4
3.0	1	1	1	1	1	2	2

TABLE C-1, continued.

TWO TAIL TEST TABLES:

For $\alpha = .01$ add 2 to the tabled value to get the number of pairs (replicates); for $\alpha = .05$ add 1 to the tabled value to get the number of pairs.

$\alpha = .01$

d \ 1-β	.50	.60	.70	.80	.90	.95	.99
.1	664	801	962	1168	1488	1782	2404
.2	166	201	241	292	372	446	601
.4	42	51	61	73	93	112	151
.6	19	23	27	33	42	50	67
.8	11	13	16	19	24	28	38
1.0	7	9	10	12	15	18	25
1.2	5	6	7	9	11	13	17
1.4	4	5	5	6	8	10	13
1.6	3	4	4	5	6	7	10
1.8	3	3	3	4	5	6	8
2.0	2	3	3	3	4	5	7
3.0	1	1	2	2	2	2	3

$\alpha = .05$

d \ 1-β	.50	.60	.70	.80	.90	.95	.99
.1	385	490	618	785	1051	1300	1838
.2	97	123	155	197	263	325	460
.4	25	31	39	50	66	82	115
.6	11	14	18	22	30	37	52
.8	7	8	10	13	17	21	29
1.0	4	5	7	8	11	12	19
1.2	3	4	5	6	8	10	13
1.4	2	3	4	5	6	7	10
1.6	2	2	3	4	5	6	8
1.8	2	2	2	3	4	5	6
2.0	1	2	2	2	3	4	5
3.0	1	1	1	1	2	2	3

Note: The tabled values are approximately values of

$u = (z_{1-\alpha} + z_{1-\beta})^2 / d^2$, where z_x denotes the cumulative distribution function of the standard normal (0,1) distribution.

Source: Experimental Statistics, by Mary G. Natrella, National Bureau of Standards Handbook 91, U. S. Government Printing Office, Washington, D.C. The tables above are Tables A-9 and A-8, respectively, from this reference.

TABLE C-2. SUMMARY STATISTICS FOR THE INTERLAB PRECISION TEST FOR DRY WEIGHT, CELL COUNT, AND LOG CELL COUNT AT 21 DAYS

		LAB 1				LAB 2			
Medium		A	B	C	D	A	B	C	D
ry Wgt.	N	5	5	5	5	5	5	5	----
	$\bar{X}$	292.4	168.4	79.28	44.08	219.42	127.14	27.56	----
	s.e.	6.89	4.93	1.94	7.87	19.23	3.47	2.41	----
	s	15.41	11.02	4.34	17.60	43.00	7.76	5.39	----
Cell No.	N	5	5	5	5	5	5	5	5
	$\bar{X}$	4656000	3152000	1908000	279200	9436000	4608000	1610000	4846
	s.e.	627301	183232.1	119725	47368	1096948	168386	63008	3521
	s	1402687	409719	267713	105918	2452850	376523	140890	7873
Log Cell No.	N	5	5	5	5	5	5	5	5
	$\bar{X}$	15.31	14.96	14.45	12.45	16.02	15.34	14.29	7.68
	s.e.	.14	.056	.063	.24	.15	.036	.039	.56
	s	.3130	.125	.1409	.537	.3354	.0805	.0872	1.25

		LAB 3				LAB 4			
		A	B	C	D	A	B	C	D
ry Wgt.	N	5	5	5	----	5	5	5	5
	$\bar{X}$	301.6	137.8	46.6	----	184.92	109	30.9	1.22
	s.e.	8.36	4.27	.98	----	22.51	1.51	5.02	.28
	s	18.69	9.55	2.19	----	50.33	3.38	11.22	.63
Cell No.	N	5	5	5	5	5	5	5	---
	$\bar{X}$	11240000	5516000	2026000	1600	7435000	3284000	1071000	---
	s.e.	400749	204490	70965	600	404784	53066	46915	---
	s	896102	457252.6	158682	1341.64	905124	118656	104904	---
Log Cell No.	N	5	5	5	5	5	5	5	---
	$\bar{X}$	16.23	15.52	14.52	7.19	15.82	15.00	13.88	---
	s.e.	.035	.038	.036	.28	.056	.016	.045	---
	s	.0783	.085	.0805	.63	.1252	.036	.1006	---

TABLE C-2, Continued.

Medium		LAB 5 A	LAB 5 B	LAB 5 C	LAB 5 D	LAB 6 A	LAB 6 B	LAB 6 C	LAB 6 D
Dry Wgt.	N	5	5	----	----	2	5	5	5
	$\bar{X}$	426.9	57.16	----	----	222.5	153.8	52.4	.60
	s.e.	15.78	2.37	----	----	12.5	6.59	1.17	.40
	s	35.28	5.30	----	----	17.68	14.74	2.62	.89
Cell No.	N	5	5	5	5	2	5	5	5
	$\bar{X}$	21260000	2153600	161580.0	8047.6	8491000	5483600	1851200	2266
	s.e.	1982509	39746	126591	1591	178000	152200.7	35440.65	47.5
	s	4433025	88875	283061	3557	251730	340331	79248	106.2
Log Cell No.	N	5	5	5	5	2	5	5	5
	$\bar{X}$	16.86	14.58	10.75	8.93	15.95	15.52	14.43	7.73
	s.e.	.091	.019	.76	.18	.021	.028	.019	.021
	s	.2035	.042	1.70	.40	.0297	.063	.0425	.047

		LAB 7 A	LAB 7 B	LAB 7 C	LAB 7 D	LAB 8 A	LAB 8 B	LAB 8 C	LAB 8 D
Dry Wgt.	N	5	4	5	5	4	5	2	----
	$\bar{X}$	218.94	146.65	39.56	6.12	377.20	164.4	55.3	----
	s.e.	10.91	4.13	1.66	3.70	10.31	3.53	7.60	----
	s	24.39	8.26	3.71	8.27	20.62	7.89	10.75	----
Cell No.	N	5	5	5	5	4	5	2	5
	$\bar{X}$	12200000	6980000	2460000	4640	30792500	8404200	2131500	4022.2
	s.e.	583095.2	332265	116619	974.5	1940818	495289.2	402500	830.6
	s	1303840	742967	260768	2179	3881636	1107500	569221	1857
Log Cell No.	N	5	5	5	5	4	5	2	5
	$\bar{X}$	16.31	15.75	14.7	8.35	17.24	15.94	14.6	8.20
	s.e.	.051	.049	.048	.21	.063	.059	.19	.23
	s	.1140	.1096	.1073	.47	.126	.132	.2687	.514